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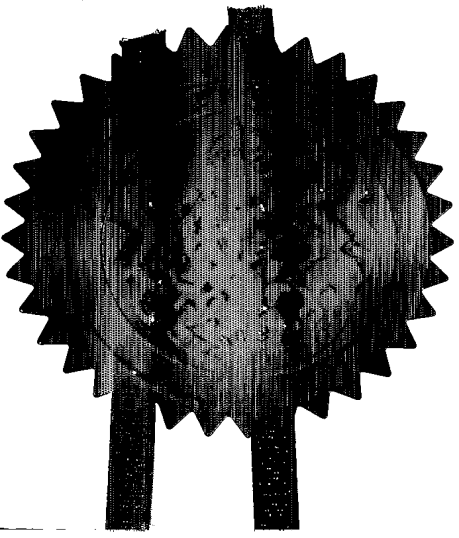

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The University of Bath
Claverton Down
Bath
BA2 7AY
United Kingdom

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P105085GB

Seeds

FIELD OF THE INVENTION

The present invention relates particularly, though not exclusively, to methods for modifying seed size in plants especially flowering plants. The invention also relates to nucleic acid constructs for use in such methods, as well as to modified plants and reproducible plant material *per se*.

BACKGROUND OF THE INVENTION

The seeds industry can be split into two high-value, commercial sectors: seeds for field crops such as corn, oil seeds, sugar beet and cereals, and vegetable and flower seed. The scientific improvement of crop plants has gone through a succession of innovations leading to the development of hybrid varieties for many crops and, most recently, to the introduction of genetically enhanced crops. The worldwide commercial seeds market is valued at around $30 billion (International Seed Federation).

1. Importance of seed size

Yield in crop plants where seed is the harvested product is usually defined as weight of seed harvested per unit area (Duvick, 1992). Consequently, individual seed weight is regarded as a major determinant of yield. Increasing seed size is desirable because it may increase total yield (Reynolds *et al.*, 2001). There is also evidence that seed size (weight) is positively correlated with a number of components of 'seed quality' such as the percentage of germination (Schaal, 1980; Alexander and Wulff, 1985; Guberac *et al,* 1998); time to emergence (Winn, 1985; Wulff, 1986); durability (survival under adverse growing conditions) (Krannitz *et al,* 1991; Manga and Yadav, 1995); and growth rate (Marshall, 1986). Seed quality is an important factor in the cost of production of commercial seed lots since these must be tested before sale. Consequently, increasing total seed weight, even without increases in total seed yield, may have economic benefits through improvements in seed quality. Conversely, decreasing seed size may also be desirable in some circumstances, for example by facilitating water uptake required for germination (Harper *et al.*, 1970), or in plants grown for their fruit.

Modification of seed size is also likely to improve yield through increasing the 'sink strength' of the seed (i.e. its capacity to demand nutrients from the seed parent), or increasing the period in which the seed is acting as a strong sink. It is well established that the demands of sink organs such as seeds have significant control over the rate of photosynthesis and the movement of photoassimilates from source to sink tissues (Patrick and Offler, 1995; Paul and Foyer, 2001). In wheat, the seed parent can supply more nutrients than developing seeds are able to demand for the first 15–20 days after pollination (Austin, 1980). Therefore modifications that enable seeds to draw nutrients earlier in development, for example by speeding up seed growth, will allow seeds to capture resources that would otherwise be wasted. An 'improved source-sink balance permitting higher sink demand during grainfilling' has also been proposed as a method for increasing yield in wheat (Reynolds *et al.*, 2001).

2. Composition of seeds

Mature seeds of flowering plants consist of three components: the seed coat, which is of exclusively maternal origin; and the two fertilization products, embryo and endosperm, which have maternal and paternal genetic contributions. Seeds develop from fertilized ovules. Ovule development has been described for many species (Bouman, 1984), including *Arabidopsis thaliana* (Robinson-Beers *et al.*, 1992; Schneitz *et al.*, 1995). The main structures of the mature ovule are: the embryo sac, which contains the female reproductive cells (egg and central cell); the nucellus, which surrounds the embryo sac at least partially; and the inner and outer integuments, which envelop the embryo sac and nucellus. After fertilization the embryo and nutritive endosperm develop inside the embryo sac while the integuments differentiate into the seed coat, which expands to accommodate the growing endosperm and embryo.

Most monocotyledonous plants, e.g. cereals including maize, wheat, rice, and barley (see Esau, 1965), produce albuminous seeds—that is, at maturity they contain a small embryo and a relatively massive endosperm. Most dicotyledonous plants, e.g. *Brassica napus,* (oil seed rape, canola), soybean, peanut, *Phaseolus vulgaris* (e.g. kidney bean, white bean, black bean) *Vicia faba* (broad bean), *Pisum sativum* (green pea), *Cicer aeietinum* (chick pea), and *Lens culinaris* (lentil), produce exalbuminous seeds—that is, the mature seeds lack an endosperm. In such seeds the embryo is large and generally fills most of the volume of the

seed, and accounts for almost the entire weight of the seed. In exalbuminous seeds the endosperm is ephemeral in nature and reaches maturity when the embryo is small and highly immature (usually heart/torpedo stage). Commonly embryo development depends on the presence of the endosperm, which is generally accepted to act as a source of nutrition for the embryo.

3. Control of seed size

Seed size control can be viewed from the perspective of (1) 'development' - the extent of cell division and expansion in one or more seed components (e.g. Reddy and Daynard, 1983; Swank *et al,* 1987; Scott *et al.*, 1998; Garcia *et al.*, 2003) or (2) 'metabolism'- metabolic activity and transport of nutrients within the seed and between the seed and seed parent (e.g. Weber *et al.*, 1996, 1997). Development and metabolism are interdependent: for example, invertase activity (involved in hexose transport) at the boundary of maternal tissues and endosperm or embryo sac is required for endosperm proliferation in maize (Cheng *et al.*, 1996), and high invertase activity is correlated with increased cell numbers in broad bean seed coat (Weber *et al.*, 1996), legume embryos (Weber *et al.*, 1997), and barley endosperm (Weschke *et al.*, 2003). Our present investigations focus on the developmental aspects of seed size control, although it can be assumed that changes to cell division/expansion in the seed will also be correlated with changes in metabolic activity and nutrient flow.

3a. Endosperm-led seed growth

Several studies show a correlation between endosperm growth and final seed size, for example in maize (Lin, 1984; Jones *et al.*, 1996), and even in the dicot *Arabidopsis thaliana*, which has an ephemeral endosperm (Scott *et al.*, 1998; Garcia *et al.*, 2003). Work in our laboratory has shown that overproliferation of the endosperm leads to large seeds with large embryos, while inhibition of endosperm proliferation produces small seeds with small embryos. We have manipulated endosperm proliferation and seed size using a variety of methods, including modifications to the ratio of paternally to maternally inherited chromosomes in the endosperm, cytosine methylation status of the parents contributing to the seed, and use of the *fis3/fie* mutation (Scott *et al.*, 1998; Adams *et al.*, 2000; Vinkenoog *et al.*, 2000). In these experiments we considered the resultant changes to seed growth to be 'endosperm-led', and effects on the embryo and the seed coat to be indirect. Some of our

experiments specifically ruled out a direct effect on seed coat growth because the seed parent was wild-type and only the fertilization products were directly modified: for example, in the case of wild-type diploid seed parents crossed with tetraploid pollen parents, which produce large seeds (Scott *et al.*, 1998), or wild-type seed parents crossed with pollen parents hypomethylated by a *DNA METHYLTRANSFERASE 1* antisense construct, which produce small seeds (Adams *et al.*, 2000). Similarly, Garcia *et al.* (2003) described the *haiku* mutants of *Arabidopsis thaliana*, which produce small seeds due to early arrest of endosperm proliferation. The authors also noted a failure of cell elongation after fertilization in the integuments of *haiku* mutants, and concluded this was an indirect effect of limited endosperm growth.

3b Role of integuments/seed coat in establishing seed size

Alonso-Blanco *et al.* (1999) investigated seed size in wild-type plants of two *Arabidopsis thaliana* accessions, Cvi and Ler: seeds of the former weigh 80% more than seeds of the latter and are 20% longer. In both accessions, the authors found that 'seed coat and endosperm growth preceded embryo growth, determining the overall final length of the embryo and the seed'. They did find that the outer layer of the mature seed coat has more cells in Cvi than Ler, but did not investigate or comment on whether these extra cells were formed before or after fertilization. Moreover, the authors' inspection of mature unfertilized ovules showed that ovules in Ler were slightly longer than in Cvi, and therefore the authors concluded that 'ovule size differences could not account for the final Ler/Cvi seed size variation'. Their overall major conclusion was that 'the larger size of Cvi seeds compared with Ler is mainly because of the faster and prolonged growth of the integuments and the endosperm' (i.e. after fertilization); they did not address the question of whether this growth was led by the integuments or the endosperm. The authors suggested that the final cell number and size in the seed coat 'may be determined during ovule development', but significantly, there was no suggestion that a larger number of integument cells before fertilization was responsible for a larger final seed size.

Weber *et al.* (1996) compared growth of the seed coat in large- and small-seeded genotypes of *Vicia faba* (broad bean). They found that large-seeded genotypes contained more cells in the seed coat at 9 days after pollination, but cell numbers in the two genotypes were similar

at 4 days after pollination. Therefore the number of cells in the integuments before fertilization could not be a factor in final seed size.

4. Relevant patent publications

(i) Fischer and Mizukami (2003), 'Methods for altering organ mass in plants', US Patent Application 20030159180

Mutations in the *AINTEGUMENTA (ANT)* gene of *Arabidopsis thaliana* prevent formation of the integuments (Klucher *et al.*, 1996; Baker *et al.*, 1997). Mizukami and Fischer (2000) describe the phenotype of *Arabidopsis thaliana* plants over-expressing the wild-type *ANT* gene under the control of the constitutive 35S promoter. Ectopic *ANT* expression increases the size of many plant organs including seeds, as well as causing male sterility through failure of anther dehiscence. Most of the transgenic plants are also female sterile 'because of abnormally extended proliferation of the chalazal nucellar cells'. However weak overexpressers could generate seeds after hand-pollination with wild-type pollen. 'The enlarged *35S::ANT* fruit included T2 seeds that were larger than normal (not shown in the application), because of enlarged embryos.' The size of unpollinated ovules, and the number or size of cells in the integuments/seed coat, were not investigated or discussed. The large seed size of *35S::ANT* seeds was attributed only to size of the nucellus and embryo. US patent application no. 20030159180 describes uses of a modified ANT polypeptide for altering the size of plant organs including seeds. It was reported that the transgenic plants had varying degrees of fertility that were not correlated with organ size. There was no investigation of the effect of expressing the modified ANT polypeptide on integument or seed coat growth.

(ii) Jofuku and Okamuro (2001), 'Methods for improving seeds', US patent 6,329,567

Mutations in the *APETALA2 (AP2)* gene increase seed size (Okamuro and Jofuku, 1997). The mutations have a maternal effect on seed size but the only phenotype described for the integument/seed coat in *ap2* mutants is that the cells of the outer layer of the seed coat are enlarged with an irregular shape, along with some other morphological abnormalities (Jofuku *et al.*, 1994). US patent no. 6,329,567 describes methods of modulating seed mass

using *AP2* transgenes, but this patent does not assess any effect of the transgenes on the integuments or seed coat.

(iii) Lepiniec *et al.* (2003), 'Regulating nucleic acid for expressing a polynucleotide of interest specifically in the endothelium of a plant seed and uses thereof', WO 03/012106 A2
The *BANYULS (BAN)* gene is expressed exclusively in the inner layer of the inner integument (this layer is also called the endothelium) in early seed development (pre-globular stage) (Devic *et al.*, 1999). International patent application WO 03/012106 A2 describes use of the *BAN* promoter to drive expression of various genes specifically in the testa (the seed coat layer derived from the inner integument). The authors propose uses such as modifying the tannin or fibre composition, or the hormonal equilibrium, but no relevant expression cassettes were reported or described. Modification of seed size is also proposed but only in the context of reducing or ablating seeds in fruit crops. A *BAN* promoter::*BARNASE* construct was shown to ablate the endothelium.

(iv) Zinselmeier *et al.* (2000), 'Regulated expression of genes in plant seeds', WO00/63401
This patent application relates to expression of genes such as *ipt* that 'affect metabolically effective levels of cytokinins in plant seeds, as well as in the maternal tissue from which such seeds arise, including developing ears, female inflorescences, ovaries, female florets, aleurone, pedicel, and pedicel-forming regions', and to transgenic plants with enhanced levels of cytokinin that exhibit 'improved seed size, decreased tip kernel abortion, increased seed set during unfavorable environmental conditions, and stability of yield'. A nucellus promoter (nucellus is the maternal tissue surrounding the embryo sac and enclosed within the integuments) is among those suggested for driving expression cassettes, but integuments are not specifically mentioned in the patent application, nor were any maternal tissue-specific expression cassettes described. The disclosure of this patent application is particularly concerned with maize.

(v) Scott (2002), 'Modified plants',. WO/0109299
This patent application relates to methods for controlling endosperm size and development through use of an antisense *DNA METHYLTRANSFERASE 1* gene that reduces cytosine methylation. As described in WO01/09299, and in Section 3a, above, modification to the cytosine methylation status of the seed or pollen parent alters seed size by altering the rate

and extent of endosperm proliferation. Therefore the disclosure of this patent application relates exclusively to 'endosperm-led' seed growth.

In summary, documents in the prior art do not include an understanding that altering the size of integuments specifically through increasing the number of cells before fertilization could affect seed size after fertilization. A small number of published papers and patent applications touch on a possible relationship between seed coat size and seed size but do not make a link between (1) integument growth pre-fertilization and (2) final seed size.

5. Integument-led seed growth

We were surprised therefore to discover in our laboratory a mutant, termed the *mnt-1* mutant, that produces enlarged seeds through a primary effect on the integuments. Specifically, we observed that the seed cavity (i.e. the space within the post-fertilization embryo sac) is longer than normal giving the embryo more space to grow as a result of an increase in cell number in the integument. This was particularly surprising in view of the earlier research mentioned in section 3a above which indicated that changes in seed growth were 'endosperm-led'. It was also surprising in view of the work of Alonso-Blanco *et al* (1999) mentioned in section 3b above which did not suggest that an increase in number of integuments cells led to an increase in seed size; and also in view of the work of Weber *et al* 1999 who found similar numbers of cells in the seed coat in small and large-seeded genotypes of broad bean soon after fertilization.

DEFINITIONS

The following non-limiting definitions of terms used in this specification are given by way of explanation.

"Nucleic acid sequence": refers to a single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases read from the 5' to the 3' end, including chromosomal DNA, plasmids, infectious polymers of DNA or RNA and DNA or RNA that performs a primarily structural role.

"Orthologues": refers to genes derived from a comon ancestral gene and which are found in different species as a result of speciation. Genes found in different species

are considered orthologues when their nucleotide sequences and/or their encoded protein sequences have a high percentage of sequence identity and/or similarity. Functions of orthologues are often highly conserved among species.

"Homologue": A gene (or protein) with a similar nucleotide (or amino acid) sequence to another gene (or protein) in the same or another species.

"Promoter": a region or sequence located upstream and/or downstream from the start of transcription involved in recognition and binding of RNA polymerase and other proteins to initiate transcription.

"Plant promoter" a promoter capable of initiating transcription in plant cells.

"Operably linked" refers to a functional linkage between a promoter and a DNA sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence. Generally, operably linked means that the nucleic acid sequences being linked are contiguous, and, where necessary to join two protein coding regions, contiguous and in the same reading frame.

"Plant": includes whole plants, plant parts, shoot vegetative organs and/or structures (e.g. leaves, stems and tubers), roots, flowers and floral organs (e.g. bracts, sepals, petals, stamens, carpels, anthers), ovules (including egg and central cells), seed (including zygote, embryo, endosperm, and seed coat), fruit (e.g., the mature ovary), seedlings, plant tissue (e.g., vascular tissue, ground tissue), cells (e.g., guard cells, egg cells, trichomes and the like), and their progeny.

"Plant cell": includes cells obtained from or found in seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The term plant cells also includes modified cells, such as protoplasts, obtained from the aforementioned tissues.

SUMMARY OF THE INVENTION

According to one aspect, the present invention provides a method of modifying cell proliferation in a plant which comprises the step of transforming a plant or plant propagating material with a nucleic acid molecule comprising at least one regulatory sequence, typically a promoter sequence, capable of directing expression within the integuments and/or seed coat of at least one nucleic acid sequence whose expression or transcription product is capable of directly or indirectly modulating cell proliferation, whereby, on expression of that sequence, cell proliferation is modified. Preferably, the overall size of the integuments/seed coat in the plant is modified. This may be useful where a product is produced in the integument/seed coat. In the preferred embodiments, this will be achieved without affecting the growth or development of any part of the plant other than the seed.

In one embodiment, the function of a gene or gene product that promotes cell division is enhanced or the function of a gene or gene product that represses cell division is inhibited. Cell division in the integuments/seed coat may be increased resulting in a larger seed compared to wild type. Ths may be advantageous because increases in seed size can be achieved which are desirable as mentioned above. The seed may be at least 15%, or 25%, larger than wild type. The number of cells in the integuments/seed coat of the plant may be increased compared to wild type. The number of cells in the integuments/seed coat of the plant may be increased by at least 30%, or 50%, compared to wild type.

Equally, the method allows the production of smaller seeds which can also be advantageous as mentioned above, and in another embodiment, the function of a gene or gene product that promotes cell division is inhibited or the function of a gene product that represses cell division is enhanced. Cell division in the integuments/seed coat may decreased resulting in a smaller seed compared to wild type. The seed may be at least 15% or 25% smaller than wild type. The number of cells in the integuments/seed cost may be decreased compared to wild type. In particular the number of cells in the integuments/seed coat may be reduced by at least 30%, or 50%, compared to wild type.

The function of a gene that modulates cell proliferation may be enhanced. Transcription of the gene is activated. Activation of transcription results in increased levels of mRNA and/or protein encoded by the gene.

A plant promoter may be operably linked to a coding region of the gene in the sense orientation. The function of the gene may be modulated by operably linking a plant promoter to a nucleic acid fragment from the gene to form a recombinant nucleic acid molecule such that an antisense strand of RNA will be transcribed.

The function of a gene may be modulated by introducing a nucleic acid fragment of the gene into an appropriate vector such that double-stranded RNA is transcribed where directed by an operably linked plant promoter. Decreased levels of mRNA and/or protein encoded by endogenous copies of the gene may be produced. Levels of mRNA and protein encoded by homologues of the gene may be reduced.

The function of the gene may be modulated by operably linking a plant promoter to a 'dominant negative' allele of the gene, which interferes with the function of the gene product.

The plant may be monocotyledonous, and is preferably a crop plant. For example, the plant may be *Tritcum spp* (wheat), *Oryza sativa* (rice), *Zea mays* (maize), *Hordeum spp.* (barley), *Secale cereale* (rye), *Sorghum bicolor* (sorghum), or *Pennisetum glaucum* (pearl millet). Alternatively the plant is dicotyledonous. For example, the plant is *Brassica napus* (oil seed rape, canola) or any other *Brassica* species used to produce oilseeds (e.g. *Brassica carinata*), *Glycine max* (soybean), *Arachis hypogaea* (peanut), *Helianthus annuus* (sunflower), *Phaseolus vulgaris* (e.g. kidney bean, white bean, black bean), *Vicia faba* (broad bean), *Pisum sativum* (green pea), *Cicer arietinum* (chick pea), *Lens culinaris* (lentil), or *Linum usitatissimum* (flax, linseed).

Integument and seed coat development is similar in all species examined in the family Brassicaceae (Bouman, 1975), to which *Arabidopsis thaliana* belongs. In *Brassica napus*, a crop plant closely related to *Arabidopsis thaliana*, the seed coat is also very similar in structure (Wan *et al.*, 2002). Therefore modifications that affect growth and development of

integuments/seed coat in *Arabidopsis thaliana* should be directly applicable to members of the Brassicaceae, including *Brassica napus*.

The mature seeds of monocots such as cereals have a distinct structure. However cereal ovules have fundamental similarities with ovules of *Arabidopsis thaliana* and other dicots, also consisting of integuments enclosing a nucellus and embryo sac.) In rice, for example, the inner integument encloses the ovule before fertilization, and its growth precedes that of the endosperm and embryo, as in *Arabidopsis thaliana* (Lopez-Dee *et al.*, 1999). Therefore modification to growth of the integuments/seed coat may also be effective in altering overall seed growth in cereal crops. Specifically in rice a modification to growth of the inner integuments may be useful in modifying seed size.

It is notable that the *INO* gene, which in *Arabidopsis thaliana* is expressed in the outer integument and required for its growth (Villanueva *et al.*, 1999), has been identified in *Nymphaea alba* (water lily), where it is also expressed in the integuments (Yamada *et al.*, 2003). As the Nymphaeaceae are basal eudicots, which are ancestral to both dicots and monocots, this suggests that the sequence and expression patterns of at least some integument genes will be conserved even among distantly related groups of flowering plants.

The present invention is complementary to the invention disclosed in WO01/09299. Modifications to endosperm-led and integument-led seed growth could be combined for an even larger effect. In some situations integument-led seed growth alone may be preferable, as it only requires modification to the seed parent, while endosperm growth is determined both by maternal and paternal contributions.

Where the regulatory sequence is a promoter, the promoter sequence may be constitutive, directing gene expression in most or all cells of the plant. An example of a constitutive promoter that may be used in some embodiments of the invention is the 35S promoter, derived from the gene that encodes the 35S subunit of Cauliflower Mosaic Virus (CaMV) coat protein. Alternatively, the promoter sequence may be specific, directing expression exclusively or primarily in one organ, tissue, or cell type of the plant. A variety of plant promoters can be used in the invention to direct expression exclusively or primarily in the

integuments or seed coat. Suitable plant promoters include those obtained from plants, plant viruses, and bacteria which comprise genes expressed in plant cells, such as Agrobacterium or Rhizobium. Some embodiments use promoters expressed in the pre-fertilization integuments. These include but are not restricted to the promoters of the following genes: *INO* (Villanueva *et al.*, 1999; At1g23420, accession no. AF195047) and *BEL1* (Reiser *et al.*, 1995; At5g41410; accession no. NM_123506). Other embodiments use promoters expressed in the seed coat after fertilization. These include but are not restricted to the promoters of the following genes: *BAN* (Devic *et al.*, 1999; At1g61720, accession no. AF092912), *TT1* (Sagasser *et al.*, 2002), *TT2* (Nesi *et al.*, 2001; At5g35550; accession no. NM_122946), *TT8* (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509), *TT12* (Debeaujon *et al.*, 2001; At3g59030, accession no. AJ294464), and *TT16* (Nesi *et al.*, 2002; At5g23260; accession no. NM_203094). Where a promoter is to be introduced into a plant, a promoter-containing nucleotide sequence of up to 2000 bp would typically be used.

The use of other regulatory sequences than a promoter to direct expression within the integuments and/or seed coat is contemplated. An example is an intron directing tissue-specific expression (see e.g. Deyholos and Sieburth, 2000).

There are a number of genes known or suspected to be involved in modulating cell proliferation, either directly or indirectly. Some embodiments of the invention use genes involved in hormone response, biosynthesis, translocation, or other aspects of hormone action. These include but are not restricted to *MNT* (described above), *IPT1* (Takei *et al.*, 2001; At1g68460, accession no. AB062607), and *ARGOS* (Hu *et al.*, 2003; At3g59900, accession no. AY305869). Other embodiments use core cell cycle genes (Vandepoele *et al.*, 2002). These include but are not restricted to *CYCD3;1* (formerly Cycδ3; Soni *et al.*, 1995; Vandepoele *et al.*, 2002; At4g34160, accession no. X83371) and *CYCB1;1* (formerly Cyc1aAt; Ferreira *et al.*, 1994; Vandepoele *et al.*, 2002; At4g37490, accession no. NM_119913). Other embodiments use transcription factors involved in regulation of the extent or rate of cell proliferation. These include but are not restricted to *ANT* (Klucher *et al.*, 1996; At4g37750, accession no. NM_119937).

An expression cassette may be used either to enhance or inhibit the function of a gene that modulates cell proliferation.

One method of enhancing function is to activate transcription of the gene, resulting in increased levels of mRNA and protein encoded by the gene. This is achieved by linking a plant promoter to the coding region of the gene (either with or without introns) in the sense orientation.

Inhibition of gene function may be achieved in several ways. One method, which uses 'antisense technology', is to link a plant promoter to a nucleic acid segment from the desired gene such that the antisense strand of RNA will be transcribed (see e.g. Branen *et al.*, 2003; Choi *et al.*, 2003). Another method, which uses 'RNAi technology', is to link a plant promoter to a nucleic acid segment from the desired gene and place the resulting recombinant nucleic acid into an appropriate vector such that double-stranded RNA is transcribed (Wang and Waterhouse, 2001). Both of these techniques may result in decreased levels of mRNA and protein encoded by the endogenous copies of the gene. A nucleic acid fragment for antisense or RNAi technology may also be designed to decrease levels of mRNA and protein encoded by homologues or orthologues of the gene. A third method of inhibiting gene function is to link a plant promoter to a 'dominant negative' allele of the gene, which interferes negatively with the function of the gene product (see e.g. Hemerly *et al.*, 1995; Nahm *et al.*, 2003). In the case of inhibition of genes (e.g., by antisense, or the use of RNAi technology) it will be recognized that the inserted polynucleotide sequence need not be identical, but may be only "substantially identical" to a sequence of the gene from which it was derived.

Alternatively, the nucleic acid sequence is a mutant form of an auxin response factor encoding gene, or a construct that inhibits expression or function of an auxin response factor. The auxin response factor gene may be *MNT* in the case of *Arabidopsis thaliana* or its orthologues in other species. In the case of *Brassica napus* the gene may be *BnARF2* as used in example 2 below. In the case of rice the gene may be *OsARF2*. The *mnt-1* mutant phenotype shows that the wild-type function of the *MNT* gene is to repress cell division in the integuments. Therefore inhibition of endogenous *MNT* expression or function may result in larger integuments and a larger seed, while enhancement of *MNT* expression or function may result in a smaller seed.

In some embodiments of the invention, cell division in the integuments/seed coat will be increased, resulting in a larger seed compared to wild type. This may be achieved by enhancing function of a gene or gene product that promotes cell division, or inhibiting function of a gene or gene product that represses cell division. In other embodiments, cell division in the integuments/seed coat will be decreased, resulting in a smaller seed. This may be achieved by enhancing function of a gene or gene product that represses cell division, or inhibiting function of a gene or gene product that promotes cell division. In these embodiments the gene or gene product may be *MNT* or an orthologue of *MNT*; alternatively it may be another gene or gene product that affects cell division.

A plant may be further modified to maintain desirable characteristics may have been otherwise lost as a result of the transformation step. For example, the desirable characteristic may be fertility.

For example, in further embodiments of the invention, *MNT* function is restored to petals and stamens of an *mnt* mutant such that seeds have the enlarged *mnt*-1 mutant phenotype but fertility is not impaired. This may be achieved by operably linking the promoter of a gene that directs expression in petals and stamens but not carpels (which contain the ovules), such as AP3 (Jack *et al.*, 1992), to the wild-type *MNT* gene. In different species, different wild type genes may be supplied.

According to another aspect of the invention there is provided a plant which includes a nucleic acid molecule comprising at least one regulatory sequence capable of directing expression within the integuments and/or seed coat of at least one nucleic acid sequence whose expression or transcription product is capable of directly or indirectly modulating cell proliferation, whereby, on expression of that sequence, cell proliferation is modified. The plant may have been obtained by a method in accordance with the invention and will have the resulting features in terms of genetic structures and phenotype as set out in any of claims 50 to 99 and/or described above.

According to a further aspect of the invention there is provided reproducible or propagatable plant material including a nucleic acid molecule comprising at least one regulatory sequence capable of directing expression within integuments and/or seed coat and at least one nucleic

acid sequence whose expression or transcription product is capable of directly or indirectly modulating cell proliferation, whereby on expression of that nucleic acid sequence cell proliferation is modified.

According to another aspect of the invention, there is provided a method of modifying cell proliferation in a plant which comprises the step of modulating the response of the plant to an auxin whereby the overall cell number of the integuments/seed coat of the plant is modified. The response to an auxin may be modified by altering the expression of an auxin response factor. Preferably, the auxin response factor is *ARF2*. The function of a gene encoding the auxin response factor may be modulated so as to affect the function of the factor. In the case of *Arabidopsis thaliana*, the gene may be *MNT*. In the case of *Brassica napus* the gene may be *BnARF2*. In the case of rice the gene may be *OsARF2*. Orthologues of these genes may be used in other species.

Most preferably, the function of an endogenous auxin response factor encoding gene is modulated for example by RNAi technology as described above. Most preferably, the function of that gene in the integuments/seed coat is affected.

Brief Description of the drawings

Embodiments in accordance with the invention will now be described, by way of example only, with reference to the accompanying drawings Figures 1 to 22 in which:

Figure 1A *Top:* Confocal micrographs of seeds with globular stage embryos from *mnt-1* (left) and wild-type (right) seed parents; *Bottom:* Mature seeds and embryos from *mnt-1* mutants and wild-type plants, photographed at the same scale;

Figure 1B is a scatter plot of number of seeds in each pod produced by *mnt-1* mutants vs mean seed weight in that pod, following controlled pollinations;

Figure 1C shows seeds from manually pollinated *mnt-1* and wild-type plants, and reciprocal crosses between them, photographed at the same scale;

Figure 2A shows light micrographs of mature unfertilized ovules, stage 3-VI (staging as in Schneitz *et al.*, 1995), from wild-type (left) and *mnt-1* (right) plants;

Figure 2B shows graphs showing number of cells, total length, and mean cell length for several integument layers in *mnt-1* and wild-type stage 3-VI ovules. (The width is also shown for layer ii1').

Figure 3 shows micrographs of the chalazal endosperm in developing seeds of *Arabidopsis thaliana*, all at the same scale. The *mnt-1* seed is an example of integument-led growth while the 2x X 6x seed provides an example of endosperm-led growth;

Figure 4A shows micrographs of *Arabidopsis thaliana* seeds illustrating endosperm-led seed growth illustrated by interploidy crosses in the C24 accession of *Arabidopsis thaliana* (see also Scott *et al.*, 1998);

Figure 4B shows micrographs illustrating integument-led seed growth illustrated by the *mnt-1* mutant in the Columbia accession of *Arabidopsis thaliana*;

Figure 4C is a micrograph of a seed illustrating the 'big bag' hypothesis;

Figure 5 is a photograph illustrating a comparison of floral phenotype and seed size in wild-type Col-3 (**5A**), *mnt-1* mutants (**5B**), and a Salk insertion mutant (Salk line 108995) homozygous for an insertion in the *ARF2* gene (**5C**);

Figure 5D is a photograph of a gel showing PCR-based scoring of segregants for the T-DNA insertion in Salk line 108995;

Figure 5E is a photograph of a gel showing scoring of presence of the insertion (top) and presence of homozygotes (bottom) in F1 progeny of the cross between an *mnt-1* homozygous mutant seed parent and the Salk 108995 homozygous pollen parent. All F1 progeny have a single copy of the insertion;

Figure 5F is a photograph illustrating floral and seed phenotype in an F1 hybrid plant resulting from a cross between a homozygous *mnt-1* mutant and a homozygous Salk insertion mutant (Salk line 108995);

Figure 6 is an alignment of wild-type *MNT* and mutant *mnt-1* cDNAs from translational start to stop;

Figure 7 is an alignment of wild-type MNT and mutant mnt-1 predicted proteins;

Figure 8 is an alignment of *Arabidopsis thaliana MNT* cDNA with its orthologue in *Brassica napus*, *BnARF2*;

Figure 9 is an alignment of *Arabidopsis thaliana* MNT predicted protein with its orthologues in *Brassica napus* (oilseed rape) (BnARF2) and *Oryza sativa* (rice) (OsARF2);

Figure 10 illustrates the BJ60, BJ40, pFGC5941, pART7, and BJ36 vectors used for the cloning strategies described in the following examples;

Figure 11 illustrates a cloning strategy for constructing reporter vectors (Example 3). In this and following figures, only restriction sites significant to the strategy are shown on the diagrams;

Figure 12 is a micrograph of a globular stage seed from a plant containing the *TT12::uidA* construct assayed for GUS expression; the inner layer of the inner integument is stained (arrow);

Figure 13 illustrates a cloning strategy for constructing an RNAi vector to constitutively decrease *MNT* expression (Example 4);

Figure 14 illustrates a cloning strategy for constructing an RNAi vector to constitutively decrease *BnARF2* expression (Example 5);

Figure 15 illustrates a cloning strategy for constructing RNAi vectors to decrease *MNT* expression primarily in the integuments/seed coat (Example 6);

Figure 16 illustrates a cloning strategy for constructing RNAi vectors to decrease *BnARF2* expression primarily in the integuments/seed coat (Example 7);

Figure 17 illustrates a cloning strategy for constructing vectors for constitutive expression of *MNT* (Example 8) or *BnARF2* (Example 9);

Figure 18 illustrates a cloning strategy for constructing vectors for expression of *MNT* in the integuments/seed coat (Example 10);

Figure 19 illustrates a cloning strategy for constructing vectors for expression of *BnARF2* in the integuments/seed coat (Example 11);

Figure 20 illustrates a cloning strategy for constructing vectors for expression of genes promoting cell division in the integuments/seed coat (Examples 12, 13);

Figure 21 is a series of photographs illustrating seed sizes and weights from individual primary transformants containing expression cassettes designed to increase seed size (*TT8::CYCD3;1* and *TT8::IPT1*) compared with controls (*TT8::uidA*). Data is taken from Table 2. Seeds were photographed at the same scale; and

Figure 22 illustrates a cloning strategy for constructing a vector for expression of *MNT* in petals and stamens (Example 14).

DESCRIPTION OF PREFERRED EMBODIMENTS

Methods and products in accordance with the present invention will now be described with reference to the following examples, which should not be construed as in any way limiting the invention.

The following vectors are used in the examples:

pGEMT (Promega, Southampton, UK)

BJ36, BJ40, BJ60 (gift of Bart Janssen, Horticultural & Food Research Institute of New Zealand)

pART7 (Gleave, 1992)

pFGC5941 (Cambia, Canberra, Australia; ChromDB, http://www.chromdb.org/plasmids)

Plant transformation protocols are based on Clough and Bent (1998) for *Arabidopsis thaliana* and Moloney *et al.* (1989) for Brassica.

Protein predictions and sequence alignments are carried out with GeneDoc software version 2.6.001 (Nicholas and Nicholas, 1997).

A. Identification of the *mnt-1* mutant

We identified the mutant, *megaintegumenta-1* (*mnt-1*), in a screen for large seeds yielded by a population of EMS (ethyl methanesulfonate)-mutagenized *Arabidopsis thaliana* in the Col-3 accession. Mature seeds produced by a seed parent homozygous for the *mnt-1* mutation are larger and more pointed than wild-type, with extra cells in the seed coat, and contain larger embryos (Fig. 1A). Specifically, Fig 1A shows that *mnt-1* mutants produce larger seeds with more cells in the seed coat (counts are for ii1, the outer layer of the inner integument).

Seeds collected from self-pollinated *mnt-1* mutant plants are up to twice the weight of wild-type Col-3 seeds (Table 1A).

Table 1A Seed weights in μg from *mnt-1* and w.t. Col-3 crosses, self seed, all siliques left on plant

	***mnt* self**	**w.t. self**
	27.1 (n=60)	14.4 (n=178)
	27.9 (106)	15.8 (75)
	29.7 (127)	
Mean	28.2	15.1
Range	27.1 to 29.7	14.4 to 15.8
Standard error	0.8	1.4

n = number of seeds weighed from each plant

However, *mnt-1* mutant plants are self-sterile until late in development due to floral abnormalities (see below), raising the possibility that the mutant produces large seeds because there are few seeds requiring maternal resources. Therefore we also conducted controlled pollinations in which only three siliques (seed pods) were allowed to set seed per plant, for both *mnt-1* and wild-type (Table 1B).

Table 1B Seed weights in µg_ from *mnt*-1 and w.t. Col-3 crosses, manual pollinations, 3 siliques per plant

	mnt* X *mnt	***mnt* X w.t.**	**w.t. X *mnt***	**w.t. X w.t.**
	31.1 (n=32)	30.4 (n=36)	30.5 (n=20)	30.6 (n=34)
	37.7 (37)	32.6 (22)	28.2 (30)	29.7 (34)
	31.4 (37)	37.4 (43)	28.2 (32)	22.1 (49)
	39 (20)	31.8 (17)	27.1 (46)	31.5 (31)
	33.7 (50)	34.4 (33)	26.5 (44)	29.6 (28)
	40.5 (22)	36 (50)	28 (44)	26.9 (53)
	38.3 (9)	37.1 (49)	30.3 (44)	30.2 (61)
	39.1 (11)	38 (24)		31.7 (48)
	37.2 (25)			21.2 (32)
	35.2 (24)			
	35.7 (43)			
	39 (31)			
	38.3 (64)			
	34 (23)			
	36.1 (33)			
	35.1 (54)			
Mean	36.3	34.7	28.4	28.7
Range	31.1 to 40.5	31.8 to 38.0	26.5 to 30.5	22.1 to 31.7
Standard error	0.7	1.0	0.6	1.3

n = number of seeds in silique

ttest [*mnt* X *mnt*] vs [w.t. X w.t.]: P < 0.000, significant

ttest [*mnt* X *mnt*] vs [*mnt* X w.t.]: P > 0.2, not significant

ttest [w.t. X w.t.] vs [w.t. X *mnt*]: P > 0.9, not significant

This treatment raised the mean weight of wild-type seeds by 90% and *mnt-1* seeds by 29%, indicating that low seed number is a component of large seed size in *mnt-1* mutants but that the *mnt-1* mutation also has a significant effect. On average *mnt-1* seeds weighed 26% more than wild-type when only three siliques per plant set seed; the difference in weights was significant at P<0.000. We also investigated whether occasional low seed set within

individual *mnt-1* siliques might raise seed weight; however a scatter plot (Fig. 1B) of mean seed weight of each pod vs number of seeds per pod following controlled pollinations in *mnt-1* (data from Table 1B) shows no correlation.

The *mnt-1* mutation has a maternal effect on seed size. That is, an *mnt-1* homozygous mutant seed parent yields large seeds regardless of whether it is pollinated by an *mnt-1* or wild-type plant, while a wild-type parent yields normal seeds even if pollinated by an *mnt-1* plant (Fig. 1C). In Fig 1C seeds produced by *mnt-1* seed parents are shown on top and seeds from wild-type seed parents are below. H = fertilization products (embryo and endosperm) are heterozygous for the *mnt*-1 mutation. This shows that seed size in *mnt-1* mutants depends on the genotype of the seed parent, not the fertilization products.

The primary difference between *mnt-1* and wild-type seeds is that the mutant seeds contain more cells in the seed coat. Comparison of ovule development in *mnt-1* and wild-type plants shows that *mnt-1* ovules are of normal size and morphology until they are near maturity, at which time we observe that both the inner and outer integuments of *mnt*-1 ovules are significantly longer than in wild-type, primarily due to a significantly greater number of cells (Fig. 2). In relation to the results depicted in Fig. 2, in *Arabidopsis thaliana* and other members of the Brassicacea most cell division and expansion occurs in the integuments on the abaxial side of the ovule (marked on wild-type ovule in Fig. 2A). Similarly, the nucellus in rice is enveloped by the abaxial inner integument (Lopez-Dee *et al.*, 1999). In Arabidopsis, ii1, ii1', and ii2 are the three cell layers of the inner integument and oi1 and oi2 are the two layers of the outer integument. The cells of layer ii1', which does not completely span the embryo sac, significantly expand in width after fertilization as part of seed growth (Beeckman *et al.*, 2000). *mnt-1* ovules have longer integuments with extra cells and in some cases an extra layer (arrow), as well as a larger seed cavity (Fig. 2A). C = the 'curving zone' of the abaxial outer integument (the region overlying ii1'; Beeckman *et al.*, 2000), M = the 'micropylar zone', regions delimited with black bars (Fig. 2A). Measurements shown in Fig. 2B were taken for the abaxial integuments only. Layers ii1', ii1, and the curving zone of oi2 are longer in *mnt-1* mutant ovules, almost exclusively due to greater cell number. Mean cell length is greater in the micropylar zone of *mnt-1* ovules but

smaller or not significantly different in the oi2 curving zone and the other integument layers measured. There is no difference in mean width between *mnt-1* and wild-type cells of layer ii1'.

The peripheral endosperm in *mnt-1* mutant seeds also generates more nuclei than in wild-type seeds. The mean number of peripheral endosperm nuclei in *mnt-1* seeds at the heart stage is 1150, compared with 550 for a wild-type Col-3 seed at a comparable stage; see Scott *et al.* (1998) for a description of endosperm morphology and the counting method. However, we consider there are two crucial differences between *mnt-1* mutant seeds and large seeds that show endosperm-led growth. First, the chalazal region of the endosperm, which becomes greatly enlarged in endosperm-led seeds (e.g. seeds from interploidy crosses generating paternal excess, crosses where the DNA of the seed parent is hypomethylated, or *fis* mutants), is of roughly normal size in *mnt-1* mutants (although the pinched shape of the chalazal pole of mnt-1 seeds results in a longer and narrower chalazal endosperm) (Fig 3). Second, the size difference between *mnt-1* and wild-type seeds follows from differences existing before fertilization i.e. before the endosperm has been created. The overproliferation of peripheral endosperm may follow from the larger seed volume created by enlarged integuments/seed coat.

B.The 'big bag' hypothesis

We observe that seeds with enlarged endosperms and seeds with large seed coats have a feature in common: the seed cavity (i.e. the space within the post-fertilization embryo sac) is larger than normal, giving the embryo more space to grow (Fig. 4A, 4B). Specifically, endosperm-led seed growth is illustrated by interploidy crosses in the C24 accession of *Arabidopsis thaliana* (see also Scott *et al.*, 1998). As shown in Fig. 4A extra paternal genomes produce seeds with a large cavity (top left, 2x X 6x cross), and ultimately large seeds with large embryos (2x X 4x cross, bottom left). Conversely, extra maternal genomes generate seeds with small cavities (top right, 6x X 2x cross), and ultimately small seeds with small embryos (4x X 2x cross, bottom right). The control 2x X 2x cross is shown in the middle.

In contrast in integument-led seed growth as illustrated in Fig. 4B the seeds also have a large seed cavity (top left) compared with wild-type (top right). Mature seeds and embryos are compared below.

This leads to our 'big bag' hypothesis, which states that seed and ultimately embryo size is set by the size of the seed cavity, which may be controlled by several factors including extent of endosperm proliferation and extent of integument/seed coat proliferation (Fig. 4C).

It is well established in the literature that after fertilization in *Arabidopsis thaliana* there is no further division in the seed coat, and growth occurs only by cell expansion (Léon-Kloosterziel *et al.*, 1994; Beeckman *et al.*, 2000; Windsor *et al.*, 2000). Obviously seeds with large endosperms must also have large seed coats; however, in this case, the seed coat grows by cell expansion after fertilization. In seeds where large seed coat is considered the primary cause of seed enlargement (integument-led seed growth), the integument/seed coat contains extra cells, as observed in *mnt*-1 mutants.

C. Further aspects of the *mnt-1* mutant phenotype

The *mnt-1* mutation affects floral morphology as well as seed size. Most flowers fail to open; this is mainly due to a lack of petal cell expansion, although there are also fewer petal cells. Additionally, pollen is shed from the anthers on to the sides of the carpel rather than the stigma due to impaired extension of stamen filaments. Filaments have a reduced number of cells, which also fail to expand normally.

The floral phenotypes result in sterility of plants unless manual pollination is carried out (*mnt-1* homozygotes are female fertile, and the pollen that completes development is also fertile). However the last few flowers produced by *mnt-1* mutants appear wild-type and these are self-fertile.

Germination frequency of *mnt-1* seeds is normal, and the seedlings are vigorous.

D. Molecular characterization of the wild-type *MNT* gene and *mnt-1* mutant allele

i) Wild-type MNT sequence

We mapped the *MNT* locus to a 60.9 kb region of chromosome 5 that was annotated by The Arabidopsis Information Resource (TAIR) (http://www.arabidopsis.org) to contain 17 genes. T-DNA insertion lines with insertions in these genes generated by The Salk Institute Genome Analysis Laboratory (SIGnAL) (Alonso *et al.*, 2003) (http://signal.salk.edu) were obtained from the Nottingham Arabidopsis Stock Centre (NASC) (http://nasc.nott.ac.uk). Salk line no.108995 (NASC stock no. N608995), with an insertion in the coding region of the *AUXIN RESPONSE FACTOR 2 (ARF2)* gene, included a plant homozygous for the insertion with a similar phenotype to *mnt-1* mutants, including closed flowers and large seeds (Fig. 5A–C). Genotypic scoring of segregants from the Salk 108995 family, including one heterozygote and the homozygote, is shown in Fig. 5D. Specifically in Fig. 5D *Top:* Scoring for presence of an insertion in the *ARF2* gene. Primers used were 5' TGG TTC ACG TAG TGG GCC ATC G 3', and 5' GAG TGG GTG GAG TGT GTT TG 3'. Lanes M and O show presence of the insertion. *Bottom:* Scoring for homozygous insertion mutants. Primers used were 5'GAG TGG GTG GAG TGT GTT TG 3' and 5' AGT TGG TTT TCG TTT GAG CAT 3'. PCR conditions are set so that the gene will only amplify if there is no insertion: therefore PCR products will be amplified from DNA extracted from wild-type plants and also those hemizygous for the insertion, but not homozygous plants. Lane M shows no amplification, indicating this plant is homozygous for the insertion. An allelism test was conducted by crossing a seed parent homozygous for the *mnt-1* mutation with the Salk 108995 homozygote as pollen parent. F1 progeny were hemizygous for the insertion (Fig. 5E) and had the *mnt-1* mutant phenotype (Fig. 5F), confirming that *MNT* is the *ARF2* gene.

MNT/ARF2 will be referred to as *MNT* in the remainder of this document. The *MNT* gene = At5g62000, accession no. NM_125593. The genomic DNA for *MNT*, including the coding region plus 4371 bases of 5' and 525 bases of 3' flanking region, is shown in SEQ ID NO. 1. SEQ ID NO. 2 is the complete cDNA, and SEQ ID NO. 3, the predicted protein.

ARFs form part of the system for responding to auxin, a hormone known to be involved in many plant developmental processes including cell division and expansion (Stals and Inzé, 2001; Leyser, 2002). ARFs are transcription factors that in general are not induced by auxin themselves but which regulate expression of auxin-inducible genes, such as members of the Aux/IAA class (Liscum and Reed, 2002). ARFs have been shown to bind to Auxin Response Elements (AREs) containing the motif TGTCTC in the promoters of auxin-inducible genes (Ulmasov *et al.*, 1999a). Twenty-two ARFs predicted to be functional have been annotated in the *Arabidopsis thaliana* genome (Hagen and Guilfoyle, 2002). ARFs contain two conserved domains—an N-terminal DNA binding domain and a C-terminal dimerization domain—and a variable middle region. An ARF may activate or repress transcription of its targets and this is thought to depend on the sequence of the middle region (Ulmasov *et al.*, 1999b). Evidence so far suggests that ARF2 is likely to be a repressor (Tiwari *et al.*, 2003).

ii) Mutant mnt-1 sequence

We sequenced the coding region from genomic DNA of the *mnt-1* allele plus 4371 bases of the 5' and 525 bases of the 3' flanking regions (this genomic sequence is shown in SEQ ID NO. 4). A single base change with respect to the wild-type Col-3 sequence, from G to A, was identified at position 665 from translational start, at the end of intron 3. This was predicted to affect splicing by changing the conserved 3' splice site (Brown and Simpson, 1998) from the consensus AG sequence to AA. We sequenced the first 837 bases of the *mnt-1* cDNA from start of translation and confirmed that four bases are deleted from the beginning of exon 4. The *mnt-1* cDNA from translational start to stop, consisting of the 837 directly sequenced bases plus the remainder of the cDNA coding region as predicted from the sequenced *mnt-1* genomic DNA, is shown in SEQ ID NO. 5. Wild-type *MNT* and mutant *mnt-1* cDNA sequences are aligned in Fig. 6.

The predicted mnt-1 protein (SEQ ID NO. 6) has a frameshift from amino acid position 123 and an early stop codon at position 167. Wild-type MNT and mutant mnt-1 predicted protein sequences are aligned in Fig. 7. The frameshift and early stop codon are both within the DNA binding domain and therefore the mnt-1 allele is likely to cause a complete loss of MNT function.

Example 1

Value of *mnt* mutants in understanding and modifying growth of integuments/seed coat.

The *mnt* mutant seed phenotype demonstrates that there is a correlation between the size of integuments before fertilization and the size of the mature seed in *Arabidopsis thaliana* (Figs 1, 2). Due to the similarities in seed structure among even distantly related groups of flowering plants, this leads to the expectation that modification to integument/seed coat size in other species, and certainly in members of the Brassicaceae such as *Brassica napus*, will also result in changes to seed size.

Our knowledge of the *mnt* mutant phenotype and *MNT* gene sequence can be exploited in other species through TILLING ('Targeting induced local lesions in genomes'). In this reverse genetics technique, chemically mutagenized populations are screened for presence of a point mutation in a nucleic acid sequence of interest; this can be done as a high-throughput procedure and is applicable to many species (Till *et al.*, 2003). For example, TILLING could be applied to the *Brassica napus* or rice orthologues of *MNT* in order to modify seed size in these crop species.

Example 2 Modifying expression of *MNT* orthologues in other species.

Knowledge of the *MNT* sequence in *Arabidopsis thaliana* also allows us to search for orthologues in crop species as a necessary first step in targeted modification of the expression of the gene in these species.

By way of example, we amplified the putative *Brassica napus* orthologue (*BnARF2*) of *MNT* using primers (SEQ ID NO 7, 8) based on the *MNT* sequence and on publicly available *Brassica oleracea* sequence. The *BnARF2* cDNA was amplified from total RNA isolated from seedlings of *Brassica napus* var. Westar. The *BnARF2* cDNA from translational start to shop is shown in SEQ ID NO. 9 and is aligned with *Arabidopsis thaliana MNT* cDNA in Fig. 8. The BnARF2 predicted protein (SEQ ID NO. 10) has 85% identity to *Arabidopsis thaliana* MNT.

A family of ARFs has also been characterized in rice and one of these, OsARF2 (accession no. AB071293), is considered to be the orthologue of *Arabidopsis thaliana* ARF2 (Sato *et al.*, 2001). Fig. 9 shows an alignment of the predicted protein sequences of MNT (*Arabidopsis thaliana* ARF2), BnARF2, and OsARF2.

Example 3

Construction, transformation, and analysis of reporter vectors to show where integument/seed coat promoters are expressed in *Arabidopsis thaliana*.

This is to test which promoters are suitable for driving integument/seed coat-specific or -preferred expression of nucleic acids such as *MNT* antisense or RNAi constructs, or other genes modifying cell proliferation.

Diagrams of the BJ60, BJ40, pFGC5941, pART7, and BJ36 vectors used in the cloning strategies described in this and following examples are shown in Fig. 10.

The cloning strategy is shown in Fig.11.

3a Construction of reporter vectors

3a(i) *TT8*

A reporter vector based on the promoter of the *TT8* gene (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509) is constructed as described below. A 1.7 kb fragment including the *TT8* promoter is amplified by the polymerase chain reaction (PCR) from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *TT8* gene using the primers TT8F and TT8R which introduce an NdeI and a PstI site at the 5' and 3' ends of the *TT8* PCR fragment respectively.

5' AAACATATGCCAACGGGATCATGGGATTAC 3' **TT8F** SEQ ID NO. 11
NdeI

5' AAACTGCAGCGTTCCCGGAGATACGAAAAC 3' **TT8R** SEQ ID NO. 12
PstI

The *TT8* PCR fragment is A-tailed and ligated into pGEMT, then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ60, 5' to the uidA reporter which includes a terminator signal, forming the vector TT8-BJ60.

3a(ii) *TT12*

A reporter vector based on the promoter of the *TT12* gene (Debeaujon *et al.*, 2000; At3g59030, accession no. AJ294464) is constructed as described below. A 1.7 kb fragment including the *TT12* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *TT12* gene using the primers TT12F and TT12R which introduce an NdeI and a PstI site at the 5' and 3' ends of the *TT12* PCR fragment respectively.

5' AAACATATGGGAATTCACAATCGGAAAGTC 3' **TT12F** SEQ ID NO. 13
NdeI

5' AAACTGCAGGGTCCGTTTATTAGTTCCTC 3' **TT12R** SEQ ID NO. 14
PstI

The *TT12* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ60, 5' to the uidA reporter gene forming TT12-BJ60.

3b Construction of binary vectors and transformation into Arabidopsis thaliana

Reporter cassettes are excised with NotI from the following vectors:

TT8-BJ60
TT12-BJ60

and ligated into the NotI sites of the binary vector BJ40, forming the following vectors for transformation:

TT8-uidA-BJ40

TT12-uidA-BJ40

The binary vectors are transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

3c Analysis of expression patterns

The *uidA* gene encodes β-glucuronidase (GUS), which is assayed using standard protocols (e.g. Jefferson, 1987). For Figure 12 (below) the following assay was used. Seeds were dissected from siliques into GUS staining buffer (100mM Tris-HCl pH 7.2, 50 mM NaCl, 0.1% Triton-X-100, 2 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucoronic acid (X-Gluc), 2mM $K_3Fe(CN_6)$, 2 mM $K_4Fe(CN)_6$) and incubated overnight at 37° C.

Figure 12 shows a globular stage seed from a plant containing the *TT12::uidA* construct assayed for GUS expression; the inner layer of the inner integument is stained (arrow), indicating activity of the *TT12* promoter fragment in that integument.

Example 4

Construction and transformation of an RNAi cassette that decreases *MNT* expression in *Arabidopsis thaliana*, including decreased expression in the integuments/seed coat.

The cloning strategy is shown in Fig. 13.

4a Construction of RNAi cassette

An RNAi vector based on the *MNT* gene (see above) is constructed as described below. A 0.57 kb fragment of the *MNT* cDNA ('*MNT*i') is amplified by PCR from *Arabidopsis thaliana* cDNA using the primers FARF2i and RARF2inew which introduce XbaI and AscI sites at the 5' end of the *MNT*i PCR fragment, and BamHI and SwaI sites at the 3' end of the PCR fragment.

5' GATCTAGAGGCGCGCCGGATCTGAGAACTGGATG 3' **FARF2i** SEQ ID NO.

15

XbaI AscI

5' GAGGATCCATTTAAATCCGCAGCATCATTCAAGT 3' **RARF2inew** SEQ ID NO. 16

BamHI SwaI

The *MNT*i PCR fragment is A-tailed and ligated into pGEMT, and then excised with AscI and SwaI and ligated into the AscI and Swal sites of the pFGC5941 RNAi vector 3' to the *35S* promoter and 5' to the *CHSA* intron, which places the fragment in forward orientation. This forms the vector 35S-MNTi-pFGC5941. The *MNT*i PCR fragment is then excised from pGEMT with BamHI and XbaI and ligated into the BamHI and XbaI sites of the 35S-MNTi-pFGC5941 vector, 3' to the CHSA intron and 5' to the *ocs* terminator signal, which places the fragment in inverse orientation. This forms the vector 35S-MNTi-inv MNTi-pFGC5941.

4b Transformation into Arabidopsis thaliana

Vector 35S-MNTi-inv MNTi-pFGC5941 is transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

Example 5

Construction and transformation of an RNAi cassette that decreases BnARF2 expression in *Brassica napus*, including decreased expression in the integuments/seed coat.

The cloning strategy is shown in Fig 14.

5a Construction of RNAi cassette

An RNAi vector based on the *BnARF2* gene (Example 2, above) is constructed as described below. A 0.56 kb fragment of the *BnARF2* cDNA (*BnARF2*i) is amplified by PCR from *Brassica napus* cDNA using the primers FBnARF2i and RBnARF2i which introduce XbaI

and AscI sites at the 5' end of the *BnARF2*i PCR fragment, and BamHI and SwaI sites at the 3' end of the PCR fragment.

5'GATCTAGAGGCGCGCCGCGATATGAGAACTGGATA 3' **FBnARF2i** SEQ ID NO. 17

XbaI AscI

5'GAGGATCCATTTAAATGTAGGCCCCGCAGGGTCA 3' **RBnARF2i** SEQ ID NO. 18

BamHI SwaI

The *BnARF2*i PCR fragment is A-tailed and ligated into pGEMT and then excised with AscI and SwaI and ligated into the AscI and SwaI sites of the pFGC5941 RNAi vector 3' to the *35S* promoter and 5' to the *CHSA* intron using the enzymes AscI and SwaI, which places the fragment in forward orientation. This forms the vector 35S-BnARF2i-pFGC5941. The *BnARF2i* PCR fragment is then excised from pGEMT with BamHI and XbaI and ligated into the BamHI and XbaI sites of the 35S-BnARF2i-pFGC5941 vector 3' to the CHSA intron and 5' to the *ocs* terminator, which places the fragment in inverse orientation. This forms the vector 35S-BnARF2i-inv BnARF2i-pFGC5941.

5b Transformation

Vector 35S-BnARF2i-inv BnARF2i-pFGC5941 is transformed into *Agrobacterium tumefaciens* and then into *Brassica napus*.

Example 6

Construction and transformation of RNAi cassettes that decrease *MNT* expression primarily in the integuments/seed coat of *Arabidopsis thaliana*.

This is specifically to phenocopy the big seed effect of *mnt* mutations without other effects on plant growth, development, or fertility.

The cloning strategy is shown in Fig. 15.

6a Construction of RNAi vectors containing an integument/seed coat promoter

6a(i) TT8

An RNAi vector in which the *TT8* promoter (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509) drives an inverted repeat of an *MNT* nucleic acid fragment (see Example 4, above) is constructed as described below. A 1.7 kb fragment including the *TT8* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *TT8* gene using the primers TT8 EcoRI F and TT8 NcoI R which introduce an EcoRI and an NcoI site at the 5' and 3' ends of the *TT8* PCR fragment respectively.

5' GAATTCCCAACGGGATCATGGGATTAC 3' **TT8Fi** SEQ ID NO. 19
EcoRI

5' CCATGGCGTTCCCGGAGATACGAAAAC 3' **TT8Ri** SEQ ID NO. 20
NcoI

The *TT8* PCR fragment is A-tailed and ligated into pGEMT, and then excised with EcoRI and NcoI and exchanged for the *35S* promoter in the vector 35S-*MNT*-inv *MNT*i-pFGC5941 (Example 4, above), forming the vector TT8-*MNT*-inv *MNT*i-pFGC5941.

6a(ii) INO

An RNAi vector in which the *INO* promoter (Villanueva *et al.*, 1999; At1g23420, accession no. AF195047) drives an inverted repeat of an *MNT* nucleic acid fragment (see Example 4, above) is constructed as described below. A 1.5 kb fragment including the *INO* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *INO* gene using the primers FINOi and RINOi which introduce an EcoRI and an NcoI site at the 5' and 3' ends of the *INO* PCR fragment respectively.

5' GAATTCCCTGGATTAGTGCAAGCC 3' **FINOi** SEQ ID NO. 21
EcoRI

5' CCATGGGAGAGTGTGTGTGTACGATG 3' **RINOi** SEQ ID NO. 22
NcoI

The *INO* PCR fragment is A-tailed and ligated into pGEMT, and then excised with EcoRI and NcoI and exchanged for the *35S* promoter in the vector 35S-MNT-inv MNTi-pFGC5941 (Example 4, above), forming the vector INO-MNT-inv MNTi-pFGC5941.

6b Transformation into Arabidopsis thaliana

The TT8-MNT-inv MNTi-pFGC5941 and INO-MNT-inv MNTi-pFGC5941 vectors are transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

Example 7

Construction and transformation of RNAi cassettes that decrease BnARF2 expression primarily in the integuments/seed coat of *Brassica napus*.

The cloning strategy is shown in Fig. 16.

7a Construction of RNAi vectors containing an integument/seed coat promoter

7a(i) TT8

An RNAi vector in which the *TT8* promoter (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509) drives an inverted repeat of a *BnARF2* nucleic acid fragment (see Example 5, above) is constructed as described below. A 1.7 kb fragment including the *TT8* promoter with EcoRI and NcoI linkers is amplified by PCR from *Arabidopsis thaliana* genomic DNA as described in Example 6a(i) above.

The *TT8* PCR fragment is A-tailed and ligated into pGEMT, and then excised with EcoRI and NcoI and exchanged for the *35S* promoter in the vector 35S-BnARF2-inv BnARF2i-pFGC5941 (Example 5, above), forming the vector TT8-BnARF2-inv BnARF2i-pFGC5941.

7a(ii) INO

An RNAi vector in which the *INO* promoter (Villanueva *et al.*, 1999; At1g23420, accession no. AF195047) drives an inverted repeat of a *BnARF2* nucleic acid fragment (see Example 5, above) is constructed as described below. A 1.5 kb fragment including the *INO* promoter with EcoRI and NcoI linkers is amplified by PCR from *Arabidopsis thaliana* genomic DNA as described in Example 6a(ii) above.

The *INO* PCR fragment is A-tailed and ligated into pGEMT, and then excised with EcoRI and NcoI and exchanged for the *35S* promoter in the vector 35S-BnARF2-inv BnARF2i-pFGC5941 (Example 5, above), forming the vector INO-BnARF2-inv BnARF2i-pFGC5941.

7b Transformation into Brassica napus

The TT8-BnARF2-inv BnARF2i-pFGC5941 and INO-BnARF2-inv BnARF2i-pFGC5941 vectors are transformed into *Agrobacterium tumefaciens* and then into *Brassica napus.*

Example 8

Construction and transformation of an expression vector that increases *MNT* expression in *Arabidopsis thaliana*, including increased expression in the integuments/seed coat.

This is to produce a plant with smaller seeds.

The cloning strategy is shown in Fig. 17.

8a Construction of a vector for constitutive expression of MNT

Construction of an expression vector with the CaMV *35S* promoter driving the *MNT* gene is described below. The *MNT* cDNA including the translational start and stop is amplified by PCR from *Arabidopsis thaliana* cDNA using the primers 35S Xho new and 35S Bam new which introduce a XhoI and a BamHI site at the 5' and 3' ends of the *MNT* PCR fragment respectively.

5' CTCGAGGAAGGTATGGCGAGT 3' **35S Xho new** SEQ ID NO. 23
XhoI

5' GGATCCTCCAGTCTCCACCAA 3' **35S Bam new** SEQ ID NO. 24
BamHI

The *MNT* PCR fragment is A-tailed and ligated into pGEMT, and then excised with XhoI and BamHI and ligated into the XhoI and BamHI sites of pART7, 3' to the *35S* promoter and 5' to the *ocs* terminator, forming the vector 35S-MNT-pART7.

8b Construction of binary vectors and transformation into Arabidopsis thaliana

The 35S::*MNT* expression cassette (including the *ocs* terminator signal) is excised from 35S-MNT-pART7 with NotI and ligated into the NotI sites of the binary vector BJ40, forming the vector 35S-MNT-BJ40. The binary vector is transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

Example 9

Construction and transformation of an expression cassette that increases *BnARF2* expression in *Brassica napus*, including increased expression in the integuments/seed coat.

This is also to produce a plant which produces smaller seeds.

The cloning strategy is shown in Fig. 17.

9a Construction of a vector for constitutive expression of BnARF2

Construction of an expression vector with the CaMV *35S* promoter driving the *BnARF2* gene is described below. The *BnARF2* cDNA from translational start to stop is amplified by PCR from *Brassica napus* cDNA using the primers BnARF2 XhoI F and BnARF2 BamHI R which introduce a XhoI and a BamHI site at the 5' and 3' ends of the *BnARF2* PCR fragment respectively.

5' CTCGAGATGGCGAGTTCGGAGGTTTC 3' **BnARF2 XhoI F** SEQ ID NO. 25
XhoI

5' GGATCCTTAAGAGTTTCCGGCGCTGG 3' **BnARF2 BamHI R** SEQ ID NO. 26
BamHI

The *BnARF2* PCR fragment is A-tailed and ligated into pGEMT, and then excised with XhoI and BamHI and ligated into the XhoI and BamHI sites of pART7, 3' to the *35S* promoter and 5' to the *ocs* terminator, forming the vector 35S-BnARF2-pART7.

9b Construction of binary vectors and transformation into Brassica napus

The *35S::BnARF2* expression cassette (including the *ocs* terminator signal) is excised from 35S-BnARF2-pART7 with NotI and cloned into the NotI sites of the binary vector BJ40, forming the vector 35S-BnARF2-BJ40.

The binary vector is transformed into *Agrobacterium tumefaciens* and then into *Brassica napus*.

Example 10

Construction and transformation of expression cassettes that increase *MNT* expression primarily in the integuments/seed coat of *Arabidopsis thaliana*.

The cloning strategy is shown in Fig. 18.

10a Construction of expression vectors containing an integument/seed coat promoter

10a(i) *TT8*

An expression vector based on the *TT8* promoter (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509) is constructed as described below. A 1.7 kb fragment including the *TT8* promoter with NdeI and PstI linkers is amplified by PCR from *Arabidopsis thaliana* genomic DNA as described in Example 3a(i), above. The *TT8* PCR fragment is A-tailed and

ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector TT8-BJ36.

10a(ii) *INO*

An expression vector based on the promoter of the *INO* gene (Villanueva *et al.*, 1999; At1g23420, accession no. AF195047) is constructed as described below. A 1.5 kb fragment including the *INO* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *INO* gene using the primers INOF and INOR which introduce an NdeI and a PstI site at the 5' and 3' ends of the INO PCR fragment respectively.

5' CATATGCCTGGATTAGTGCAAGGCAA 3' **INOF** SEQ ID NO. 27
NdeI

5' CTGCAGGAGAGTGTGTGTGTACGATG 3' **INOR** SEQ ID NO. 28
PstI

The *INO* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector INO-BJ36.

10b Construction of expression vectors containing a promoter::MNT expression cassette

The *MNT* cDNA with XhoI and BamHI linkers is amplified by PCR from *Arabidopsis thaliana* cDNA and ligated into pGEMT as described in Example 8a, above.

10b(i) *TT8-MNT*

The *MNT* PCR fragment is excised from pGEMT with XhoI and BamHI and ligated into the XhoI and BamHI sites of the TT8-BJ36 vector, 3' to the *TT8* promoter, forming the vector TT8-MNT-BJ36.

10b(ii) *INO-MNT*

The *MNT* PCR fragment is excised from pGEMT with XhoI and BamHI and ligated into the XhoI and BamHI sites of the INO-BJ36 vector, 3' to the *INO* promoter, forming the vector INO-MNT-BJ36.

10c Construction of binary vectors and transformation

10c(i) *TT8-MNT*

The *TT8::MNT* expression cassette (including the *ocs* terminator signal) is excised from TT8-MNT-BJ36 with NotI and cloned into the NotI sites of the binary vector BJ40, forming the vector TT8-MNT-BJ40.

10c(ii) *INO-MNT*

The *INO::MNT* expression cassette (including the *ocs* terminator signal) is excised from INO-MNT-BJ36 with NotI and cloned into the NotI sites of the binary vector BJ40, forming the vector INO-MNT-BJ40.

The TT8-MNT-BJ40 and INO-MNT-BJ40 binary vectors are transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

Example 11

Construction and transformation of expression vectors that increase *BnARF2* expression primarily in the integuments/seed coat of *Brassica napus*.

The cloning strategy is shown in Fig. 19.

11a Construction of expression vectors containing an integument/seed coat promoter

11a(i) *TT8*

An expression vector based on the promoter of the *TT8* gene (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509) is constructed as described below. A 1.7 kb fragment including the *TT8* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *TT8* gene using the primers TT8F and TT8 MluI R which introduce an NdeI and an MluI site at the 5' and 3' ends of the *TT8* PCR fragment respectively.

5' AAACATATGCCAACGGGATCATGGGATTAC 3' **TT8F** SEQ ID NO. 11

NdeI
5' AAAACGCGTCGTTCCCGGAGATACGAAAAC 3' **TT8 MluI R** SEQ ID NO. 29
MluI

The *TT8* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and MluI and ligated into the NdeI and MluI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector TT8 (NdeI MluI)-BJ36.

11a(ii) *INO*

An expression vector based on the promoter of the *INO* gene (Villanueva *et al.*, 1999; At1g23420, accession no. AF195047) is constructed as described below. A 1.5 kb fragment including the *INO* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *INO* gene using the primers INOF and INO MluI R which introduce an NdeI and an MluI site at the 5' and 3' ends of the *INO* PCR fragment respectively.

5' CATATGCCTGGATTAGTGCAAGGCAA 3' **INOF** SEQ ID NO. 27
NdeI
5' ACGCGTGAGAGTGTGTGTCTACGATG 3' **INO MluI R** SEQ ID NO. 30
MluI

The *INO* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and MluI and ligated into the NdeI and MluI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector INO (NdeI MluI)-BJ36.

11b Construction of expression vectors containing a promoter::BnARF2 expression cassette

The *BnARF2* cDNA with XhoI and BamHI linkers is amplified by PCR from *Brassica napus* cDNA and ligated into pGEMT as described in Example 9a, above.

11b(i) *TT8*

The *BnARF2* PCR fragment is excised from pGEMT with XhoI and BamHI and ligated into the XhoI and BamHI sites of the TT8 (NdeI MluI)-BJ36 vector, 3' to the *TT8* promoter, forming the vector TT8-BnARF2-BJ36.

11b(ii) INO

The *BnARF2* PCR fragment is excised from pGEMT with XhoI and BamHI and ligated into the XhoI and BamHI sites of the INO (NdeI MluI)-BJ36 vector, 3' to the *INO* promoter, forming the vector INO-BnARF2-BJ36.

11c Construction of binary vectors and transformation

11c(i) *TT8*

The TT8-BnARF2 expression cassette (including the *ocs* terminator signal) is excised from TT8-BnARF2-BJ36 with NotI and ligated into the NotI sites of the binary vector BJ40, forming the vector TT8-BnARF2-BJ40.

11c(ii) INO

The INO-BnARF2 expression cassette (including the *ocs* terminator signal) is excised from INO-BnARF2-BJ36 with NotI and ligated into the NotI sites of the binary vector BJ40, forming the vector INO-BnARF2-BJ40.

The binary vectors TT8-BnARF2-BJ40 and INO-BnARF2-BJ40 are transformed into *Agrobacterium tumefaciens* and then into *Brassica napus*.

Example 12

Construction, transformation, and analysis of expression vectors that increase expression of a gene promoting cell division in the integuments/seed coat of *Arabidopsis thaliana*

The cloning strategy is shown in Fig. 20.

12a Construction of expression vectors containing an integument/seed coat promoter

12a(i) *TT8*

An expression vector based on the *TT8* promoter (Nesi *et al.*, 2000; At4g09820, accession no. AJ277509) is constructed as described below. A 1.7 kb fragment including the *TT8* promoter with NdeI and PstI linkers is amplified by PCR from *Arabidopsis thaliana* genomic DNA as described in Example 3a(i), above. The *TT8* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector TT8-BJ36.

12a(ii) *TT12*

An expression vector based on the *TT12* promoter (Debeaujon *et al.*, 2000; At3g59030, accession no. AJ294464) is constructed as described below. A 1.7 kb fragment including the *TT12* promoter with NdeI and PstI linkers is amplified by PCR from *Arabidopsis thaliana* genomic DNA as described in Example 3a(ii), above. The *TT12* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector TT12-BJ36.

12a(iii) *INO*

An expression vector based on the *INO* promoter (Villanueva *et al.*, 1999; At1g23420, accession no. AF195047) is constructed as described below. A 1.5 kb fragment including the *INO* promoter with NdeI and PstI linkers is amplified by PCR from *Arabidopsis thaliana* genomic DNA as described in Example 10a(ii), above. The *INO* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector INO-BJ36.

12a(iv) BAN

An expression vector based on the promoter of the *BAN* gene (Devic *et al.*, 1999; At1g61720, accession no. AF092912) is constructed as described below. A 0.4 kb fragment including the *BAN* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *BAN* gene using the primers BANF and BANR which introduce an NdeI and a PstI site at the 5' and 3' ends of the *BAN* PCR fragment respectively.

5' CATATGGAGAATTTGACAGATTGGTG 3' **BANF** SEQ ID NO. 31
NdeI
5' CTGCAGGTTTATCGTCTTGAGACTTC 3' **BANR** SEQ ID NO. 32
PstI

The *BAN* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of BJ36, 5' to the *ocs* terminator signal, forming the vector BAN-BJ36.

12b Construction of expression cassettes containing an integument/seed coat promoter driving a gene promoting cell division

12b(i) Promoter::*CYCD3;1*

Construction of expression vectors with an integument/seed coat promoter driving the *CYCD3;1* gene is described below. The *CYCD3;1* cDNA (formerly *Cycδ3*; Soni *et al.*, 1995; Vandepoele *et al.*, 2002; At4g34160, accession no. X83371) is amplified by PCR from *Arabidopsis thaliana* cDNA using the primers CYCD3F and CYCD3R which introduce a SmaI and a BamHI site at the 5' and 3' ends of the *CYCD3;1* PCR fragment respectively.

5' AAACCCGGGATGGCGATTCGGAAGGAGGAA 3' **CYCD3F** SEQ ID NO. 33
SmaI
5' AAAGGATCCTTATGGAGTGGCTACGATTGC 3' **CYCD3R** SEQ ID NO. 34
BamHI

The *CYCD3;1* PCR fragment is A-tailed and ligated into pGEMT, and then excised with SmaI and BamHI and ligated into the SmaI and BamHI sites of the following vectors:
TT8-BJ36 vector, 3' to the *TT8* promoter and 5' to the *ocs* terminator signal, forming the vector TT8-CYCD3;1-BJ36
TT12-BJ36 vector, 3' to the *TT12* promoter and 5' to the *ocs* terminator signal, forming the vector TT12-CYCD3;1-BJ36
INO-*BJ36* vector, 3' to the *INO* promoter and 5' to the *ocs* terminator signal, forming the vector INO-CYCD3;1-BJ36

BAN-BJ36 vector, 3' to the *BAN* promoter and 5' to the *ocs* terminator signal, forming the vector BAN-CYCD3;1-BJ36

12b(ii) Promoter::*IPT1*

Construction of expression vectors with an integument/seed coat promoter driving the *IPT1* gene is described below. The *IPT1* gene (Takei *et al.*, 2001; At1g68460, accession no. AB062607) is amplified by PCR from *Arabidopsis thaliana* genomic DNA (the *IPT1* gene contains no introns) using the primers IPT1F and IPT1R which introduce a SmaI and a BamHI site at the 5' and 3' ends of the *IPT1* PCR fragment respectively.

5' AAACCCGGGATGACAGAACTCAACTTCCAC 3' **IPT1F** SEQ ID NO. 35
SmaI

5' AAAGGATCCCTAATTTTGCACCAAATGCCG 3' **IPT1R** SEQ ID NO. 36
BamHI

The *IPT1* PCR fragment is A-tailed and ligated into pGEMT, and then excised with SmaI and BamHI and ligated into the SmaI and BamHI sites of the following vectors:
TT8-BJ36 vector, 3' to the *TT8* promoter and 5' to the *ocs* terminator signal, forming the vector TT8-IPT1-BJ36
TT12-BJ36 vector, 3' to the *TT12* promoter and 5' to the *ocs* terminator signal, forming the vector TT12-IPT1-BJ36
INO-BJ36 vector, 3' to the *INO* promoter and 5' to the *ocs* terminator signal, forming the vector INO-IPT1-BJ36
BAN-BJ36 vector, 3' to the *BAN* promoter and 5' to the *ocs* terminator signal, forming the vector BAN-IPT1-BJ36

12b(iii) Promoter::*ANT*

Construction of expression vectors with an integument/seed coat promoter driving the *ANT* gene is described below. The *ANT* gene (Klucher *et al.*, 1996; At4g37750, accession no. NM_119937) is amplified by PCR from *Arabidopsis thaliana* cDNA using the primers ANTF and ANTR which introduce a SmaI and a BamHI site at the 5' and 3' ends of the ANT PCR fragment respectively.

5' CCCGGGGGTGTGTTCGTTGTGTAACC 3' **ANTF** SEQ ID NO. 37
SmaI

5' GGATCCGATCAAGAATCAGCCCAAGC 3' **ANTR** SEQ ID NO. 38
BamHI

The *ANT* PCR fragment is A-tailed and ligated into pGEMT, and then excised with SmaI and BamHI and ligated into the SmaI and BamHI sites of the following vectors:
TT8-BJ36 vector, 3' to the *TT8* promoter and 5' to the *ocs* terminator signal, forming the vector TT8-ANT-BJ36
TT12-BJ36 vector, 3' to the *TT12* promoter and 5' to the *ocs* terminator signal, forming the vector TT12-ANT-BJ36
INO-BJ36 vector, 3' to the *INO* promoter and 5' to the *ocs* terminator signal, forming the vector INO-ANT-BJ36
BAN-BJ36 vector, 3' to the *BAN* promoter and 5' to the *ocs* terminator signal, forming the vector BAN-ANT-BJ36

12b(iv) Promoter::CYCB1;1

Construction of expression vectors with an integument/seed coat promoter driving the *CYCB1;1* gene is described below. The *CYCB1;1* gene (formerly *Cyc1aAt*; Ferreira *et al.*, 1994; Vandepoele *et al.*, 2002; At4g37490, accession no. NM_119913) is amplified by PCR from *Arabidopsis thaliana* cDNA using the primers CYCB1F and CYCB1R which introduce a SmaI and a BamHI site at the 5' and 3' ends of the *CYCB1;1* PCR fragment respectively.

5' CCCGGGCACTAAGATGATGACTTCTC 3' **CB1F** SEQ ID NO. 39
SmaI

5' GGATCCAAGCGACTCATTAGACTTGT 3' **CB1R** SEQ ID NO. 40
BamHI

The *CYCB1;1* PCR fragment is A-tailed and ligated into pGEMT, and then excised with SmaI and BamHI and ligated into the SmaI and BamHI sites of the following vectors:
TT8-BJ36 vector, 3' to the *TT8* promoter and 5' to the *ocs* terminator signal, forming the vector TT8-CYCB1;1-BJ36

TT12-BJ36 vector, 3' to the *TT12* promoter and 5' to the *ocs* terminator signal, forming the vector TT12-CYCB1;1-BJ36
INO-BJ36 vector, 3' to the *INO* promoter and 5' to the *ocs* terminator signal, forming the vector INO-CYCB1;1-BJ36
BAN-BJ36 vector, 3' to the *BAN* promoter and 5' to the *ocs* terminator signal, forming the vector BAN-CYCB1;1-BJ36

12c Construction of binary vectors and transformation into Arabidopsis thaliana

Expression cassettes (including the ocs terminator) are excised with NotI from the following vectors

TT8-CYCD3;1-BJ36
TT8-IPT1-BJ36
TT8-ANT-BJ36
TT8-CYCB1;1-BJ36
TT12-CYCD3;1-BJ36
TT12-IPT1-BJ36
TT12-ANT-BJ36
TT12-CYCB1;1-BJ36
INO-CYCD3;1-BJ36
INO-IPT1-BJ36
INO-ANT-BJ36
INO-CYCB1;1-BJ36
BAN-CYCD3;1-BJ36
BAN-IPT1-BJ36
BAN-ANT-BJ36
BAN-CYCB1;1-BJ36

and ligated into the NotI sites of the binary vector BJ40, forming the following vectors for transformation:

TT8-CYCD3;1-BJ40

TT8-IPT1-BJ40
TT8-ANT-BJ40
TT8-CYCB1;1-BJ40
TT12-CYCD3;1-BJ40
TT12-IPT1-BJ40
TT12-ANT-BJ40
TT12-CYCB1;1-BJ40
INO-CYCD3;1-BJ40
INO-IPT1-BJ40
INO-ANT-BJ40
INO-CYCB1;1-BJ40
BAN-CYCD3;1-BJ40
BAN-IPT1-BJ40
BAN-ANT-BJ40
BAN-CYCB1;1-BJ40

The binary vectors are transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

12d Analysis of seed weights in transformants

Results from some primary transformants using the *TT8* promoter are shown in Table 2 and Fig. 21. The histogram shows that seeds from *TT8::CYCD3;1* and *TT8::IPT1* plants have a broader distribution and higher peak of weights than the controls. TT8::uidA lines were used as controls, as expression of the *uidA* gene is not found to affect plant growth and development. Individual *TT8::CYCD3;1* plants produced seeds up to 97% heavier than controls, with a mean increase over 27 lines of 37%. *TT8::IPT1* plants produced seeds up to 107% heavier, with a mean increase over 24 lines of 28%. The mean weights of *TT8::CYCD3;1* and *TT8::IPT1* seeds were compared with the controls using t-tests and found to be significantly different from the controls with $P<0.000$. It should be noted that some of the *TT8::IPT1* lines, including the highest weighing line, also had a vegetative phenotype including dwarfing, serrated leaves, and extremely low fertility, most likely due to the *TT8* promoter driving vegetative expression of *IPT1* in some lines. However lines with normal vegetative development also produced large seeds. It is likely that vegetative

expression of *TT8* could be prevented if required by the technique of promoter dissection (e.g. Chandrasekharan *et al.*, 2003).

13 Construction, transformation, and analysis of expression vectors that increase expression of a gene promoting cell division in the integuments/seed coat of *Brassica napus*

The binary vectors described in Example 12c (above) are transformed into *Brassica napus*.

14 Construction of an expression vector containing a sepal-and petal-specific promoter driving *MNT* and transformation into *mnt* mutants

The cloning strategy is shown in Fig. 22.

14a Construction of an expression vector based on the AP3 promoter

An expression vector based on the promoter of the *AP3* gene (Jack *et al.*, 1992; At3g54340, accession no.AY142590) is constructed as described below. A 1 kb fragment including the *AP3* promoter is amplified by PCR from *Arabidopsis thaliana* genomic DNA 5' to translational start of the *AP3* gene using the primers AP3F and AP3R which introduce an NdeI and a PstI site at the 5' and 3' ends of the *AP3* PCR fragment respectively.

5' AAACATATGGATACACAAGTTCTTTGG 3' **AP3F** SEQ ID NO. 41
NdeI

5' AAACTGCAGATTCTTCTCTCTTTGTTTAA 3' **AP3R** SEQ ID NO. 42
PstI

The *AP3* PCR fragment is A-tailed and ligated into pGEMT, and then excised with NdeI and PstI and ligated into the NdeI and PstI sites of the BJ36 vector, 5' to the *ocs* terminator signal, forming the vector AP3-BJ36.

14b Construction of an expression vector containing an AP3::MNT expression cassette

The *MNT* cDNA with XhoI and BamHI linkers is amplified by PCR from *Arabidopsis thaliana* cDNA and ligated into pGEMT as described in Example 8a, above. The *MNT* PCR fragment is excised with XhoI and BamHI and ligated into the XhoI and BamHI sites of the AP3-BJ36 vector, 3' to the AP3 promoter and 5' to the *ocs* terminator, forming the vector AP3-MNT-BJ36.

14c Construction of binary vector and transformation into Arabidopsis thaliana

The *AP3::MNT* expression cassette (including the *ocs* terminator signal) is excised from AP3-MNT-BJ36 with NotI and cloned into the NotI sites of the binary vector BJ40, forming the vector AP3-MNT-BJ40.

The binary vector is transformed into *Agrobacterium tumefaciens* and then into *Arabidopsis thaliana.*

SEQ ID NOS

1 *MNT* genomic DNA w.t Col-0

2 *MNT* cDNA w.t.Col-0

3 MNT predicted protein w.t. Col-0

4 *mnt-1* genomic DNA Col-3

5 *mnt-1* cDNA Col-3, translational start to stop

6 mnt-1 predicted protein Col-3

7 F primer for amplifying *Brassica napus ARF2* cDNA

8 R primer for amplifying *Brassica napus ARF2* cDNA

9 *BnARF2* cDNA, translational start to stop

10 *BnARF2* predicted protein

11 F primer for *TT8* promoter with NdeI linker

12 R primer for *TT8* promoter with PstI linker

13 F primer for *TT12* promoter with NdeI linker

14 R primer for *TT12* promoter with PstI linker

15 F primer for *MNT* RNAi fragment with XbaI and AscI linkers

16 R primer for *MNT* RNAi fragment with BamHI and SawI linkers

17 F primer for *BnARF2* RNAi fragment with XbaI and AscI linkers

18 R primer for *BnARF2* RNAi fragment with BamHI and SwaI linkers

19 F primer for *TT8* promoter with EcoRI linker

20 R primer for *TT8* promoter with NcoI linker

21 F primer for *INO* promoter with EcoRI linker

22 R primer for *INO* promoter with NcoI linker

23 F primer for *MNT* cDNA with XhoI linker

24 R primer for *MNT* cDNA with BamHI linker

25 F primer for *BnARF2* cDNA with XhoI linker

26 R primer for *BnARF2* cDNA with BamHI linker

27 F primer for *INO* promoter with NdeI linker

28 R primer for *INO* promoter with PstI linker

29 R primer for *TT8* promoter with MluI linker

30 R primer for *INO* promoter with MluI linker

31 F primer for *BAN* promoter with NdeI linker

32 R primer for *BAN* promoter with PstI linker

33 F primer for *CYCD3;1* cDNA with SmaI linker

34 R primer for *CYCD3;1* cDNA with BamHI linker

35 F primer for *IPT1* cDNA with SmaI linker

36 R primer for *IPT1* cDNA with BamHI linker

37 F primer for *ANT* cDNA with SmaI linker

38 R primer for *ANT* cDNA with BamHI linker

39 F primer for *CYCB1;1* cDNA with SmaI linker

40 R primer for *CYCB1;1* cDNA with BamHI linker

41 F primer for *AP3* promoter with NdeI linker

42 R primer for *AP3* promoter with PstI linker

SEQ ID NO. 1

MNT wild-type genomic DNA, Col-0

agccattttgtaactgaccaccgagtaatctgtaatctgagctcttttattaatcggattgaataaattcgcttggagtccgtcagtcgtgtcc
gtgagcgcgtgtctcactcgcttgagctgatgaagtgcgataatgacgtggcatgttgggatggagaccaaagaccagcattttatttta
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SEQ ID NO. 2

MNT complete cDNA wild-type Col-0

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SEQ ID NO. 3

MNT predicted protein wild-type Col-0

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SEQ ID NO. 4

mnt-1 genomic DNA Col-3

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SEQ ID NO. 5

mnt-1 cDNA translational start to stop

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SEQ ID NO. 6

mnt-1 predicted protein Col-3

MASSEVSMKGNRGGDNFSSSGFSDPKETRNVSVAGEGQKSNSTRSAAAER
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FLHLRGSRCIRSAKP

SEQ ID NO. 7

Forward primer for amplifying *Brassica napus ARF2*

5' ATGGCGAGTTCGGAGGTTT 3'

SEQ ID NO. 8

Reverse primer for amplifying *Brassica napus ARF2*

5' TGGACAATGAAGGATTTGATG 3'

SEQ ID NO. 9

BnARF2 cDNA, translational start to stop

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TATCCTCAGGTAGGCATGGACCTACTTGCACGGATTTGCTTTCTGGCTTT
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GTTTATCATCACCACCTTCTGTGGCTGCAAGGAAAATCCTCAGCGACCA
GGATGGCAAGTTTGAATATCTTGCTAACCAGTGGATGATGCACTCAGGC
CTTTCCCTGAAGTTACATGAATCTCCTAAAGTCCCTGCCGCATCTGATGC
CTCTTTCCAAGGGATAGGCAATCCCAATTACGGCGAATATGCTTTGCCT
CGTGCAGTGACGACTGAGAATGCTGCTGGCAACTGGCCAATACGTCCAC
GTGCTCTAAATTATTTTGAAGAAGCGGTTCATGCTCAGGCTAGAGAGCA
TGTGACAAAACGTCCTGCGGTCGTACAAGAGGAGGCAGCAAAGCCAAG
AGACGGGAACTGCAGGCTTTTTGGCATTCCTCTGGTGAACAACGTGAAT
GGGACAGATACAACTTTGTCTCAGAGAAACAATTTGAATGACCCTGCGG
GGCCTACGCAGATGGCATCACCAAAGGTTCAGGATCTTTCTGACCAGTC
CAAAGGGTCAAAATCGACAAATGATCATCGTGAGCAAGGACGACCATT
CCCGGTTAGTAAACCCCATCCGAAAGACGTTCAAACCAAAACAAACTCA
TGTAGGAGCTGCACGAAGGTTCAGAAGCAGGGGATTGCACTTGGCCGG
TCAGTGGATCTCTCAAAGTTCCAGAACTATGAGGAGTTGGTTACTGAAT
TGGATAGGCTGTTTGAGTTCAATGGAGAGTTGATGGCTCCTAAGAAAGA
TTGGCTGATAGTTTACACAGATGATGAGAATGATATGATGCTTGTTGGA
GACGATCCTTGGCAGGAGTTTTGTTGCATGGTTCGTAAAATCTTCATATA
CACGAAAGAGGAGGTCAGGAAGATGAACCCGGGAACTCTATGCTGTAG
GAACGAGGAAGAACCAGTTGTTGGGGAAGGATCAGATGCAAAGGACGC
GAAGTCTGCATCAAATCCTTCATTGTCCAGCGCCGGAAACTCTTAA

SEQ ID NO. 10

BnARF2 predicted protein

MASSEVSMKGNRGRGENFSSAGYSDPTVAGEAQKTQSNRSVAAERVVDPE
AALYRELWHACAGPLVTVPRQDDRVFYFPQGHIEQVEASTNQAAEQQMPL
YDLPSKILCRVINVDLKAEADTDEVYAQITLLPEPVQDENSIEKEAPPPPPR
FQVHSFCKTLTASDTSTHGGFSVLRRHADECLPPLDMSRQPPTQELVAKDL
HASEWRFRHIFRGQPRRHLLQSGWSVFVSSKRLVAGDAFIFLRGENGELRV
GVRRAMRQQGNVPSSVISSHSMHLGVLATAWHAISTGTMFTVYYKPRTSP
SEFIVPFDQYTESVKINYSIGMRFKMRFEGEEAPEQRFTGTIVGIEDSDPTRW
AKSKWRSLKVRWDETTSIPRPDRVSPWKIEPALSPPALSPVPMPRPKRPRSN

LASSTPDSSMRIREGSSKANMDPLPASGLSRVLQGQEYPTLRTKHVESVECD
APENSVVWQSSTDDDKVDVISASRRYENWISSGRHGPTCTDLLSGFGTNIEP
PHGHQIPFYDRLSSPPSVAARKILSDQDGKFEYLANQWMMHSGLSLKLHES
PKVPAASDASFQGIGNPNYGEYALPRAVTTENAAGNWPIRPRALNYFEEAV
HAQAREHVTKRPAVVQEEAAKPRDGNCRLFGIPLVNNVNGTDTTLSQRNN
LNDPAGPTQMASPKVQDLSDQSKGSKSTNDHREQGRPFPVSKPHPKDVQT
KTNSCRSCTKVQKQGIALGRSVDLSKFQNYEELVTELDRLFEFNGELMAPK
KDWLIVYTDDENDMMLVGDDPWQEFCCMVRKIFIYTKEEVRKMNPGTLC
CRNEEEPVVGEGSDAKDAKSASNPSLSSAGNS

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Claims

1. A method of modifying cell proliferation in a plant which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising at least one regulatory sequence capable of directing expression within the integuments and/or seed coat of at least one nucleic acid sequence whose expression or transcription product is capable of directly or indirectly modulating cell proliferation, whereby, on expression of that sequence, cell proliferation is modified.

2. A method according to claim 1 in which the overall size of the integuments/seed coat in the plant is modified.

3. A method according to claim 1 or 2 in which the function of a gene or gene product that promotes cell division is enhanced or the function of a gene or gene product that represses cell division is inhibited.

4. A method according to claim 3 in which cell division in the integuments/seed coat is increased resulting in a larger seed compared to wild type.

5. A method according to claim 4 in which the seed is at least 15% larger than wild type.

6. A method according to any preceding claim in which the number of cells in the integuments/seed coat of the plant is increased compared to wild type.

7. A method according to claim 6 in which the number of cells in the integuments/seed coat of the plant is increased by at least 30% compared to wild type.

8. A method according to claim 1 or 2 in which the function of a gene or gene product that promotes cell division is inhibited or the function of a gene product that represses cell division is enhanced.

9. A method according to Claim 8 in which cell division in the integuments/seed coat is decreased resulting in a smaller seed compared to wild type.

10. A method according to claim 9 in which the seed is at least 15% smaller than wild type.

11. A method according to claims, 8, 9 or 10 in which the number of cells in the integuments/seed cost is decreased compared to wild type.

12. A method according to claim 11 in which the number of cells in the integuments/seed coat is decreased by at least 30% compared to wild type.

13. A method according to any one of claims 1 to 12 in which growth or development of any part of the plant other than the seed is not substantially affected.

14. A method according to any preceding claim in which the regulatory sequence includes a promoter.

15. A method according to claim 14 in which the promoter is a constitutive promoter.

16. A method according to claim 15 in which the promoter directs gene expression in most or all cells of the plant.

17. A method according to claim 15 or 16 in which the promoter is the *35S* promoter.

18. A method according to claim 15 in which the promoter is specific, directing expression exclusively or primarily in one organ, tissue, or cell type of the plant.

19. A method according to claim 18 in which the promoter directs expression exclusively or primarily in the integuments or seed coat.

20. A method according to claim 19 in which the promoter is expressed in the pre-fertilization integuments.

21. A method according to claim 20 in which the promoter is the promoter of the *INO* or *BEL1* gene.

22. A method according to claim 19 in which the promoter is expressed in the seed coat after fertilization.

23. A method according to claim 22 in which the promoter is the promoter of the *BAN*, *TT1*, *TT2*, *TT8*, *TT12*, or *TT16* gene.

24. A method according to any preceding claim in which the nucleic acid sequence includes or is derived from a gene involved in hormone response, biosynthesis, translocation, or other aspects of hormone action.

25. A method according to claim 24 in which the gene is *MNT*, *IPT1*, or *ARGOS* or their orthologues.

26. A method according to any one of claims 1 to 23 in which the nucleic acid sequence includes or is derived from a core cell cycle gene.

27. A method according to claim 26 in which the core cell cycle gene is *CYCD3;1* or *CYCB1;1* or their orthologues.

28. A method according to any preceding claim in which the nucleic acid is a transcription factor involved in regulation of the extent or rate of cell proliferation.

29. A method according to claim 28 in which the transcription factor is *ANT* or its orthologue.

30. A method according to any one of claims 3 to 29 in which the function of a gene that modulates cell proliferation is enhanced.

31. A method according to claim 30 in which transcription of the gene is activated.

32. A method according to claim 31 in which activation of transcription results in increased levels of mRNA and/or protein encoded by the gene.

33. A method according to any preceding claim in which a plant promoter is operably linked to a coding region of the gene in the sense orientation.

34. A method according to any one of claims 3 to 33 in which the function of the gene is modulated by operably linking a plant promoter to a nucleic acid fragment from the gene to form a recombinant nucleic acid molecule such that an antisense strand of RNA will be transcribed.

35. A method according to claim 34, in which the function of a gene is modulated by introducing nucleic acid segments of the gene into an appropriate vector such that double-stranded RNA is transcribed where directed by an operably linked plant promoter.

36. A method according to claim 34 or 36 in which decreased levels of mRNA and/or protein encoded by endogenous copies of the gene are produced.

37. A method according to any one of claims 34 to 36 in which the levels of mRNA and protein encoded by homologues of the gene are reduced.

38. A method according to claim 19 in which the function of the gene is modulated by operably linking a plant promoter to a 'dominant negative' allele of the gene, which interferes with the function of the gene product.

39. A method according to any preceding claim in which the nucleic acid sequence comprises or is derived either from wild-type *MNT* or a mutant form of *mnt* or its orthologues.

40. A method according to any preceding claim in which the plant is further modified to maintain desirable characteristics in other parts of the plant which may have been lost or modified after the transformation step.

41. A method according to claim 40 in which the desirable characteristic is fertility.

42. A method according to claim 41 in which wild type MNT function (or function of an MNT orthologue) is restored to petals and stamens of a transformed plant or an *mnt* mutant plant such that seeds have a modified phenotype but fertility is not impaired.

43. A method according to claim 42 in which the promoter of a gene that directs expression in petals and stamens but not carpels is operably linked to the wild-type *MNT* gene or an *MNT* orthologue.

44. A method according to claim 43 in which the promoter is the promoter of the AP3 gene.

45. A method according to any preceding claim in which the plant is monocotyledonous.

46. A method according to claim 45 in which the plant is a crop plant.

47. A method according to claim 46 in which the plant is *Tritcum spp* (wheat), *Oryza sativa* (rice), *Zea mays* (maize), *Hordeum spp.* (barley), *Secale cereale* (rye), *Sorghum bicolor* (sorghum), or *Pennisetum glaucum* (pearl millet).

48. A method according to any one of claims 1 to 43 in which the plant is dicotyledonous.

49. A method according to claim 48 in which the plant is *Brassica napus* (oil seed rape, canola) or any other *Brassica* species used to produce oilseeds (e.g. *Brassica carinata*), *Glycine max* (soybean), *Arachis hypogaea* (peanut), *Helianthus annuus* (sunflower), *Phaseolus vulgaris* (e.g. kidney bean, white bean, black bean), *Vicia faba* (broad bean), *Pisum sativum* (green pea), *Cicer arietinum* (chick pea), *Lens culinaris* (lentil), or *Linum usitatissimum* (flax, linseed).

50. A plant which includes a nucleic acid molecule comprising at least one regulatory sequence capable of directing expression within the integuments and/or seed coat of at least one nucleic acid sequence whose expression or transcription product is capable of

directly or indirectly modulating cell proliferation, whereby, on expression of that sequence, cell proliferation is modified.

51. A plant according to claim 50 in which the overall size of the integuments/seed coat in the plant is modified.

52. A plant according to claim 50 or 51 in which the function of a gene or gene product that promotes cell division is enhanced or the function of a gene or gene product that represses cell division is inhibited.

53. A plant according to claim 52 in which cell division in the integuments/seed coat is increased resulting in a larger seed compared to wild type.

54. A plant according to claim 4 in which the seed is at least 15% larger than wild type.

55. A plant according to any one of claims 50 to 54 in which the number of cells in the integuments/seed coat of the plant is increased compared to wild type.

56. A plant according to claim 55 in which the number of cells in the integuments/seed coat of the plant is increased by at least 30% compared to wild type.

57. A plant according to claim 50 or 51 in which the function of a gene or gene product that promotes cell division is inhibited or the function of a gene product that represses cell division is enhanced.

58. A plant according to Claim 56 in which cell division in the integuments/seed coat is decreased resulting in a smaller seed compared to wild type.

59. A plant according to claim 58 in which the seed is at least 15% smaller than wild type.

60. A plant according to claims, 57, 58 or 59 in which the number of cells in the integuments/seed cost is decreased compared to wild type.

61. A plant according to claim 60 in which the number of cells in the integuments/seed coat is decreased by at least 30% compared to wild type.

62. A plant according to any one of claims 50 to 61 in which growth or development of any part of the plant other than the seed is not substantially affected.

63. A plant according to any one of claims 50 to 62 in which the regulatory sequence includes a promoter.

64. A plant according to claim 63 in which the promoter is a constitutive promoter.

65. A plant according to claim 64 in which the promoter directs gene expression in most or all cells of the plant.

66. A plant according to claim 64 or 65 in which the promoter is the *35S* promoter.

67. A plant according to claim 64 in which the promoter is specific, directing expression exclusively or primarily in one organ, tissue, or cell type of the plant.

68. A plant according to claim 67 in which the promoter directs expression exclusively or primarily in the integuments or seed coat.

69. A plant according to claim 68 in which the promoter is expressed in the pre-fertilization integuments.

70. A plant according to claim 64 in which the promoter is the promoter of the *INO* or *BEL1* gene.

71. A plant according to claim 68 in which the promoter is expressed in the seed coat after fertilization.

72. A plant according to claim 71 in which the promoter is the promoter of the *BAN, TT1, TT2, TT8, TT12, or TT16* gene.

73. A plant according to any one of claims 50 to 72 in which the nucleic acid sequence includes or is derived from a gene involved in hormone response, biosynthesis, translocation, or other aspects of hormone action.

74. A plant according to claim 73 in which the gene is *MNT*, *IPT1*, or *ARGOS* or their orthologues.

75. A plant according to any one of claims 50 to 72 in which the nucleic acid sequence includes or is derived from a core cell cycle gene.

76. A plant according to claim 75 in which the core cell cycle gene is *CYCD3;1* or *CYCB1;1* or their orthologues.

77. A plant according to any one of claims 50 to 76 in which the nucleis acid encodes a transcription factor involved in regulation of the extent or rate of cell proliferation.

78. A plant according to claim 77 in which the transcription factor is *ANT* or its orthologue.

79. A plant according to any one of claims 52 to 78 in which the function of a gene that modulates cell proliferation is enhanced.

80. A plant according to claim 79 in which transcription of the gene is activated

81. A plant according to claim 80 in which activation of transcription results in increased levels of mRNA and/or protein encoded by the gene.

82. A plant according to any one of claims 50 to 81 in which a plant promoter is operably linked to a coding region of the gene in the sense orientation.

83. A plant according to any one of claims 52 to 82 in which the function of the gene is modulated by introducing a nucleic acid segments of the gene into an appropriate vector such that double-stranded RNA is transcribed where directed by an operably linked plant promoter.

84. A plant according to claim 83, in which the function of the gene is modulated by operably linking a plant promoter to a nucleic acid segment from the gene, and introducing the resulting recombinant nucleic acid into an appropriate vector such that double-stranded RNA is transcribed.

85. A plant according to claim 83 or 84 in which decreased levels of mRNA and/or protein encoded by endogenous copies of the gene are produced.

86. A plant according to any one of claims 83 to 84 in which the levels of mRNA and protein encoded by homologues of the gene are reduced.

87. A plant according to claim 68 in which the function of the gene is modulated by operable links a plant promoter to a 'dominant negative' allele of the gene, which interferes with the function of the gene product.

88. A plant according to any one of claims 50 to 87 in which the nucleic acid sequence comprises or is derived either from wild-type *MNT* or a mutant form of *mnt* or its orthologues.

89. A plant according to any one of claims 50 to 88 in which the plant is further modified to maintain desirable characteristics in other parts of the plant which may have been lost or modified after the transformation step.

90. A plant according to claim 89 in which the desirable characteristic is fertility.

91. A plant according to claim 90 in which wild type MNT function (or the function of an MNT orthologue) is restored to petals and stamens of a transformed plant or an *mnt* mutant plant such that seeds have a modified phenotype but fertility is not impaired.

92. A plant according to claim 91 in which the promoter of a gene that directs expression in petals and stamens but not carpels is operably linked to a wild type *MNT* gene or an *MNT* orthologue.

93. A plant according to claim 92 in which the promoter is the promoter of the AP3 gene.

94. A plant according to any one of claims 50 to 93 in which the plant is monocotyledonous.

95. A plant according to claim 94 in which the plant is a crop plant.

96. A plant according to claim 95 in which the plant is *Tritcum spp* (wheat), *Oryza sativa* (rice), *Zea mays* (maize), *Hordeum spp.* (barley), *Secale cereale* (rye), *Sorghum bicolor* (sorghum), or *Pennisetum glaucum* (pearl millet).

97. A plant according to any one of claims 50 to 93 in which the plant is dicotyledonous.

98. A plant according to claim 97 in which the plant is *Brassica napus* (oil seed rape, canola), *Glycine max* (soybean), *Arachis hypogaea* (peanut), *Helianthus annuus* (sunflower), *Phaseolus vulgaris* (e.g. kidney bean, white bean, black bean), *Vicia faba* (broad bean), *Pisum sativum* (green pea), *Cicer arietinum* (chick pea), *Lens culinaris* (lentil), or *Linum usitatissimum* (flax, linseed).

99. A method for modifying cell proliferation in a plant which comprises the step of modulating the response of the plant to an auxin in which cell proliferation is modified to produce larger or smaller seeds than wild-type.

100. A method according to claim 99 in which the response to an auxin is modulated by altering the expression of an auxin response factor.

101. A method according to claim 100 in which the auxin response factor inhibits cell division.

102. A method according to claim 101 in which the auxin response factor inhibits cell division in the integuments/seed coat.

103. A method according to claim 102 in which the auxin response factor is ARF2.

104. A method according to any one of claims 9 to 103 in which the function or expression of the auxin response factor is inhibited.

105. A method according to claim 104 in which the function or expression of the auxiliary response factor is specifically in habited in the integument/seed coat of the plant.

106. A method according to any one of claims 99 to 105 in which a gene encoding the auxin response factor is modified so as to affect expression of the factor.

107. A method according to claim 106 in which the gene is *MNT* or an orthologue thereof.

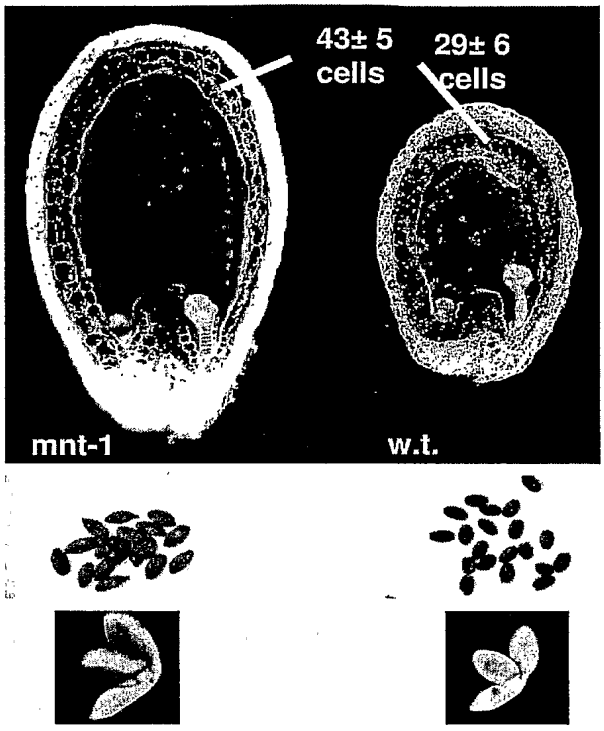
Seeds

This invention relates to a method for modifying cell proliferation in a plant which comprises the step of transforming a plant, or plant propagating material, with a nucleic acid molecule comprising at least one regulatory sequence capable of directing expression within the integuments and/or seed coat and at least one nucleic acid sequence whose expression or transcription product is capable of directly or indirectly modulating cell proliferation, whereby on expression of that nucleic acid sequence cell proliferation is modified. The invention also relates to a plant which includes a nucleic acid molecule comprising at least one regulatory sequence capable of directing expression within integuments and/or seed coat and at least one nucleic acid sequence whose expression or transcription product is capable of directly or indirectly modulated cell proliferation, whereby on expression of that nucleic acid sequence cell proliferation is modified. The invention also relates to reproducible plant material including a nucleic acid molecule comprising at least one regulatory sequence capable of directing expression within integuments and/or seed coat and at least one nucleic acid sequence whose expression or transcription product is capable of directly or indirectly modulated cell proliferation, whereby on expression of that nucleic acid sequence cell proliferation is modified.

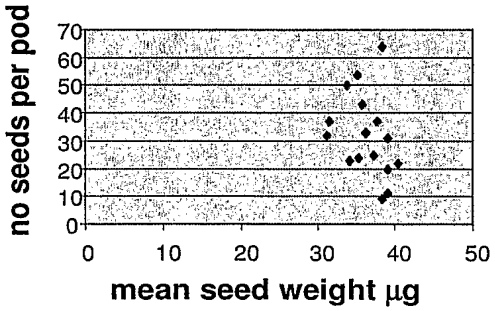
According to another aspect of the invention, there is provided a method for modifying cell proliferation in a plant which comprises the step of modulating the response of the plant to an auxin in which cell proliferation is modified to produce larger or smaller seeds than wild-type.

Figure 1

1A mnt-1 vs wild-type seeds



1B Seed weight vs no. seeds per pod in mnt-1



1C Maternal effect of mnt-1 mutation

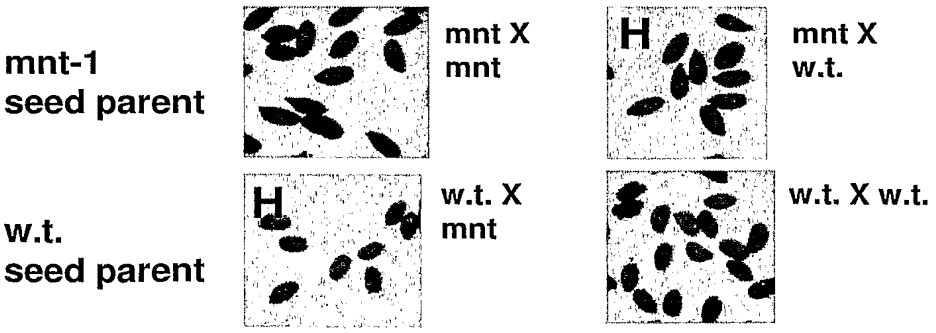
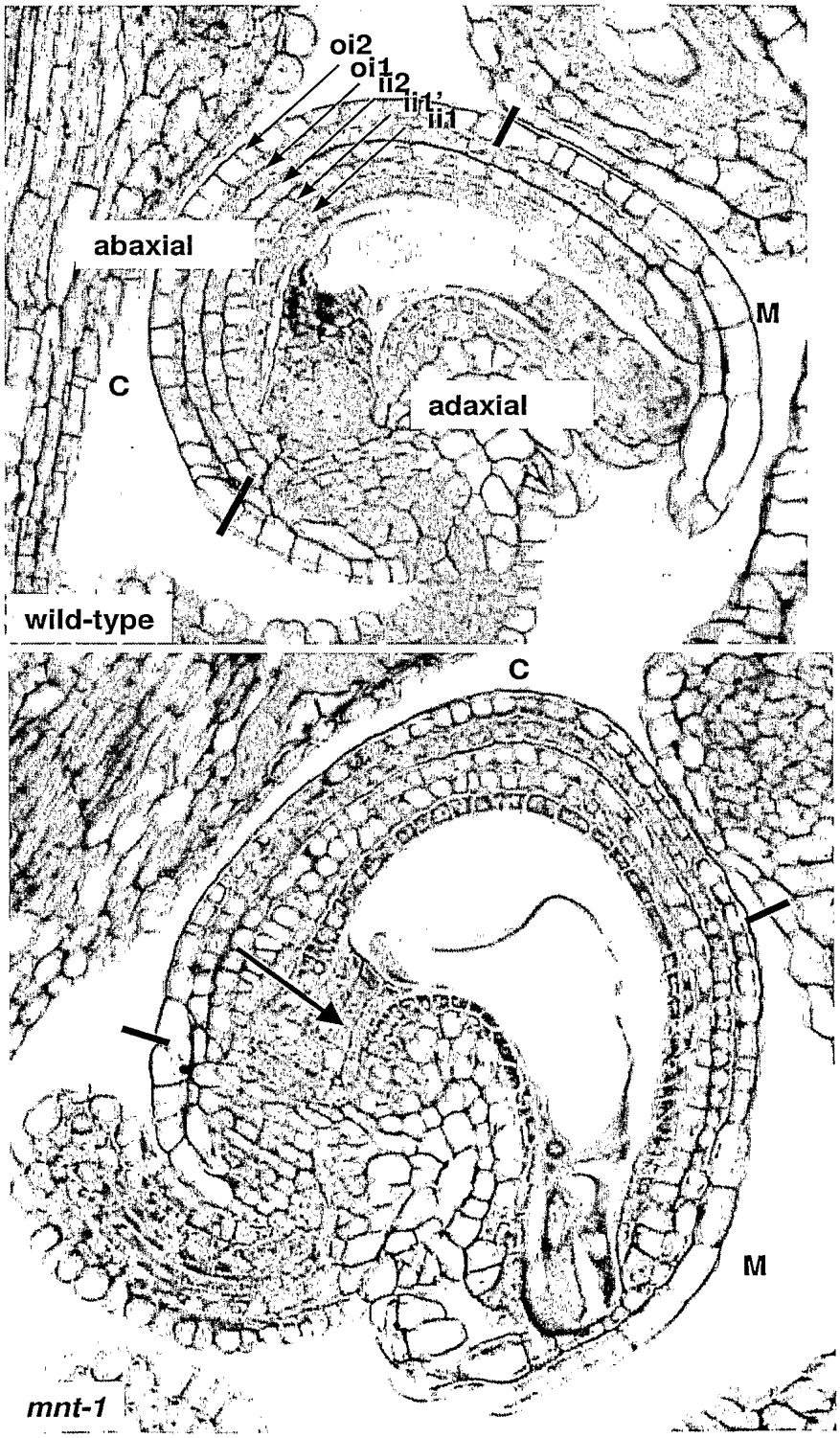
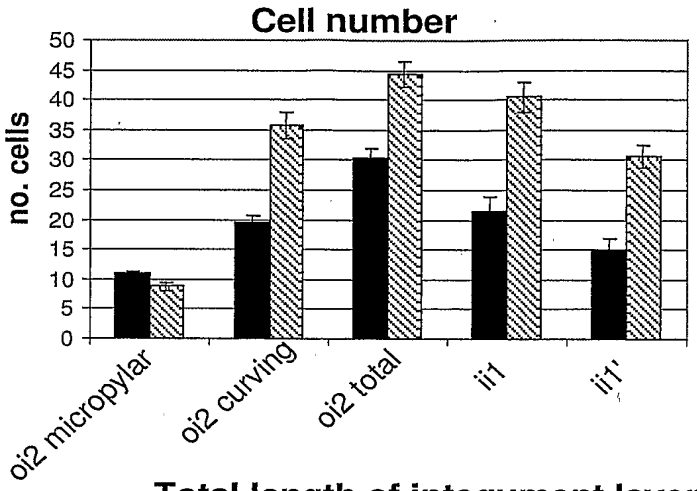


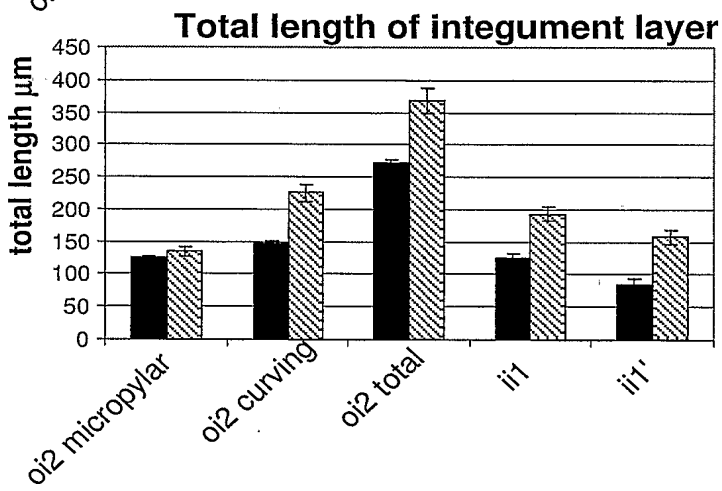
Figure 2

2A Mature w.t. and mnt-1 ovules



2B Cell number and size in w.t. and mnt-1 integuments





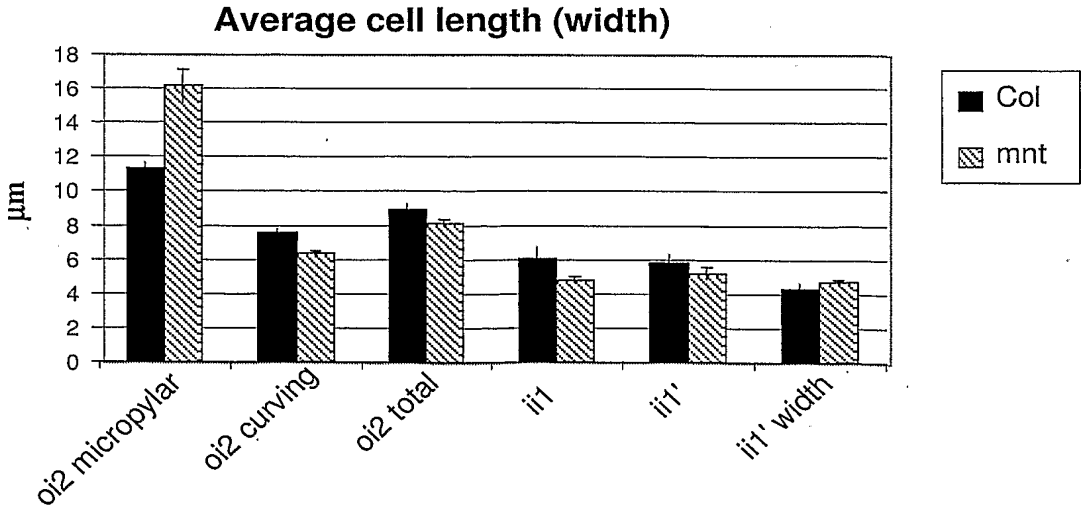
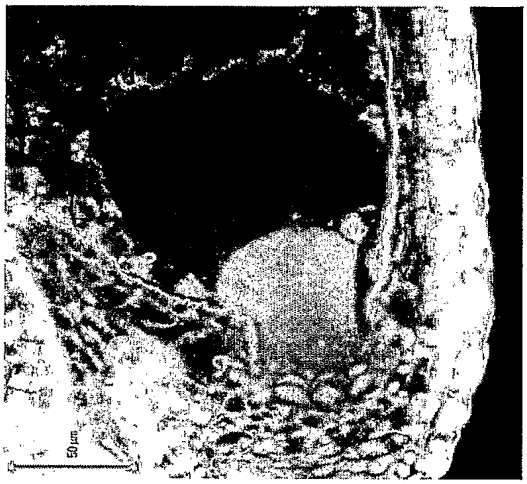


Figure 3
Chalazal endosperm



w.t. 7DAP

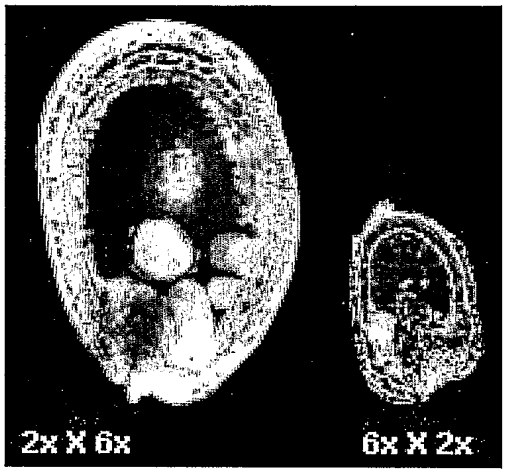
mnt-1 7DAP

2x X 6x 5 DAP

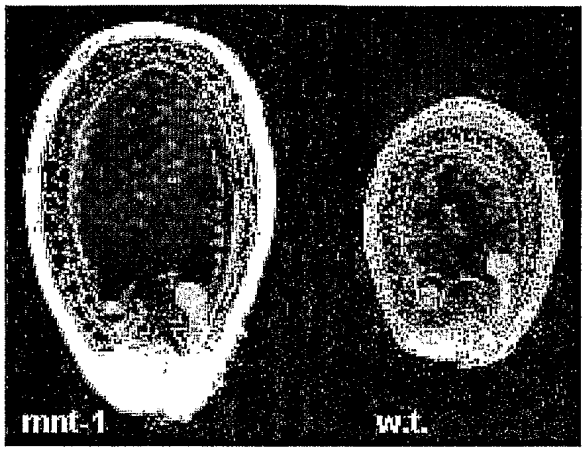
Bars = 50 µm

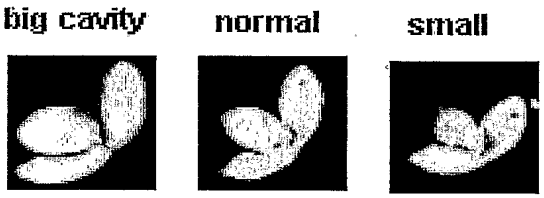
Figure 4

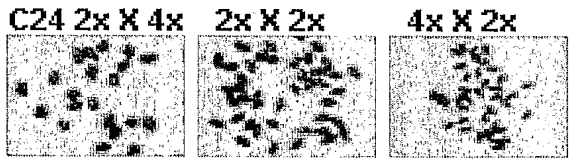
4A Endosperm-led growth

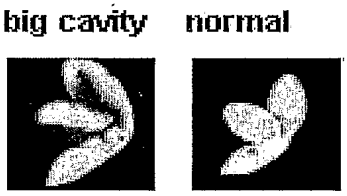


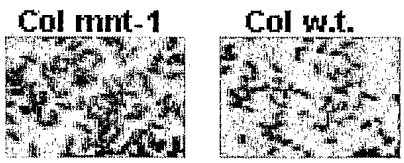
4B Integument-led growth











4C 'Big bag' hypothesis: seed and embyro size set by size of the seed cavity

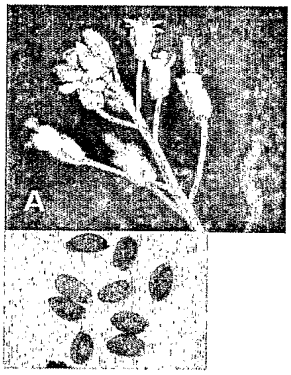
1. Division in endosperm (maternal and paternal control)

2. Division in integuments/ seed coat (maternal control)

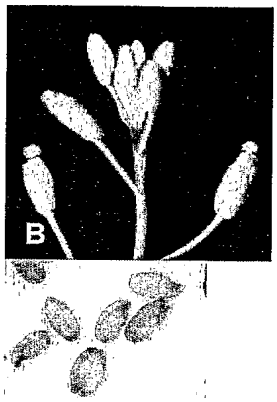
Figure 5

Allelism of mnt-1 and Salk insertion line 108995

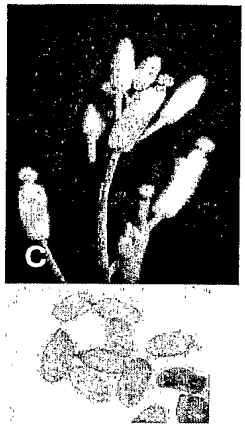
Col-3 w.t.

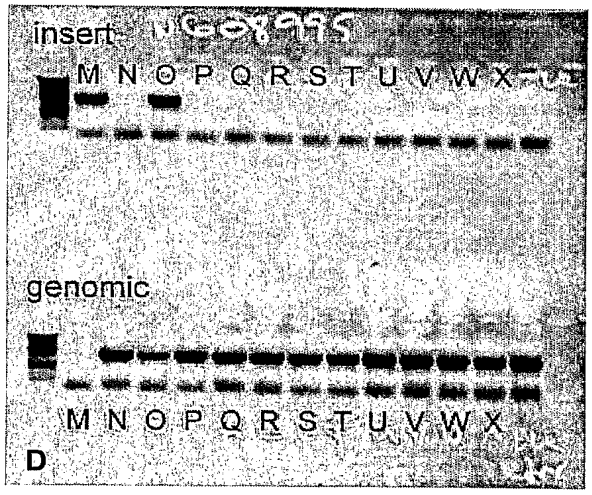


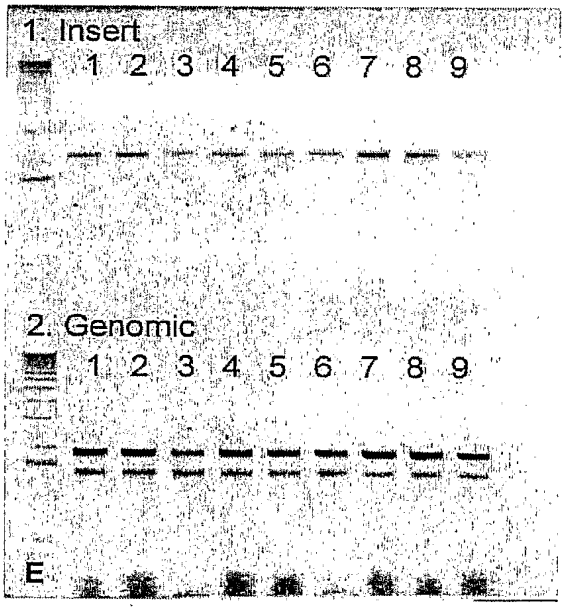
mnt-1



Salk 108995 homozygote



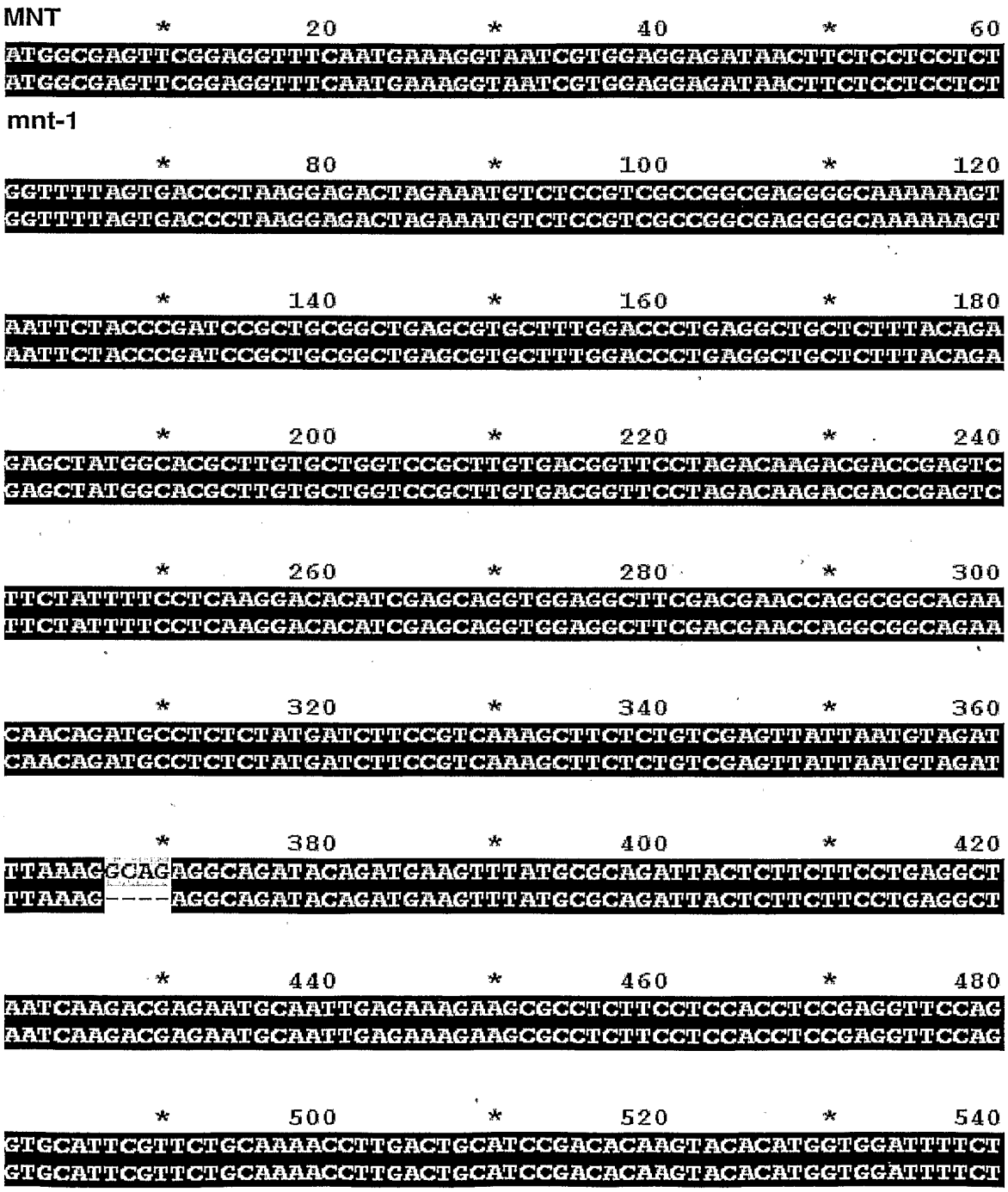




F1 mnt-1 X Salk 108995

Figure 6

Alignment of w.t. MNT and mutant mnt-1 cDNA



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          *         620         *         640         *         660
ACTCAAGAGTTAGTTGCAAAGGATTTGCATGCAAATGAGTGGCGATTCAGACATATATTC
ACTCAAGAGTTAGTTGCAAAGGATTTGCATGCAAATGAGTGGCGATTCAGACATATATTC

          *         680         *         700         *         720
CGGGGTCAACCACGGAGGCATTTGCTACAGAGTGGGTGGAGTGTGTTTGTTAGCTCCAAA
CGGGGTCAACCACGGAGGCATTTGCTACAGAGTGGGTGGAGTGTGTTTGTTAGCTCCAAA

          *         740         *         760         *         780
AGGCTAGTTGCAGGCGATGCGTTTATATTTCTAAGGGGCGAGAATGGAGAATTAAGAGTT
AGGCTAGTTGCAGGCGATGCGTTTATATTTCTAAGGGGCGAGAATGGAGAATTAAGAGTT

          *         800         *         820         *         840
GGTGTAAGGCGTGCGATGCGACAACAAGGAAACGTGCCGTCTTCTGTTATATCTAGCCAT
GGTGTAAGGCGTGCGATGCGACAACAAGGAAACGTGCCGTCTTCTGTTATATCTAGCCAT

          *         860         *         880         *         900
AGCATGCATCTTGGAGTACTGGCCACCGCATGGCATGCCATTTCAACAGGGACTATGTTT
AGCATGCATCTTGGAGTACTGGCCACCGCATGGCATGCCATTTCAACAGGGACTATGTTT

          *         920         *         940         *         960
ACAGTCTACTACAAACCCAGGACGAGCCCATCTGAGTTTATTGTTCCGTTCGATCAGTAT
ACAGTCTACTACAAACCCAGGACGAGCCCATCTGAGTTTATTGTTCCGTTCGATCAGTAT

          *         980         *        1000         *        1020
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ATGGAGTCTGTTAAGAATAACTACTCTATTGGCATGAGATTCAAAATGAGATTTGAAGGC

          *        1040         *        1060         *        1080
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GAAGAGGCTCCTGAGCAGAGGTTTACTGGCACAATCGTTGGGATTGAAGAGTCTGATCCT

          *        1100         *        1120         *        1140
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ACTAGGTGGCCAAAATCAAAGTGGAGATCCCTCAAGGTGAGATGGGATGAGACTTCTAGT
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          *       1160          *       1180          *       1200
ATTCCTCGACCTGATAGAGTATCTCCGTGGAAAGTAGAGCCAGCTCTTGCTCCTCCTGCT
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          *       1220          *       1240          *       1260
TTGAGTCCTGTTCCAATGCCTAGGCCTAAGAGGCCCAGATCAAATATAGCACCTTCATCT
TTGAGTCCTGTTCCAATGCCTAGGCCTAAGAGGCCCAGATCAAATATAGCACCTTCATCT

          *       1280          *       1300          *       1320
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CCTGACTCTTCGATGCTTACCAGAGAAGGTACAACTAAGGCAAACATGGACCCTTTACCA

          *       1340          *       1360          *       1380
GCAAGCGGACTTTCAAGGGTCTTGCAAGGTCAAGAATACTCGACCTTGAGGACGAAACAT
GCAAGCGGACTTTCAAGGGTCTTGCAAGGTCAAGAATACTCGACCTTGAGGACGAAACAT

          *       1400          *       1420          *       1440
ACTGAGAGTGTAGAGTGTGATGCTCCTGAGAATTCTGTTGTCTGGCAATCTTCAGCGGAT
ACTGAGAGTGTAGAGTGTGATGCTCCTGAGAATTCTGTTGTCTGGCAATCTTCAGCGGAT

          *       1460          *       1480          *       1500
GATGATAAGGTTGACGTGGTTTCGGGTTCTAGAAGATATGGATCTGAGAACTGGATGTCC
GATGATAAGGTTGACGTGGTTTCGGGTTCTAGAAGATATGGATCTGAGAACTGGATGTCC

          *       1520          *       1540          *       1560
TCAGCCAGGCATGAACCTACTTACACAGATTTGCTCTCCGGCTTTGGGACTAACATAGAT
TCAGCCAGGCATGAACCTACTTACACAGATTTGCTCTCCGGCTTTGGGACTAACATAGAT

          *       1580          *       1600          *       1620
CCATCCCATGGTCAGCGGATACCTTTTTTATGACCATTCATCATCACCTTCTATGCCTGCA
CCATCCCATGGTCAGCGGATACCTTTTTTATGACCATTCATCATCACCTTCTATGCCTGCA

          *       1640          *       1660          *       1680
AAGAGAATCTTGAGTGATTCAGAAGGCAAGTTCGATTATCTTGCTAACCAGTGGCAGATG
AAGAGAATCTTGAGTGATTCAGAAGGCAAGTTCGATTATCTTGCTAACCAGTGGCAGATG

          *       1700          *       1720          *       1740
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ATACACTCTGGTCTCTCCCTGAAGTTACATGAATCTCCTAAGGTACCTGCAGCAACTGAT
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GCGTCTCTCCAAGGGCGATGCAATGTTAAATACAGCGAATATCCTGTTCTTAATGGTCTA
GCGTCTCTCCAAGGGCGATGCAATGTTAAATACAGCGAATATCCTGTTCTTAATGGTCTA

          *       1820          *       1840          *       1860
TCGACTGAGAATGCTGGTGGTAACTGGCCAATACGTCCACGTGCTTTGAATTATTATGAG
TCGACTGAGAATGCTGGTGGTAACTGGCCAATACGTCCACGTGCTTTGAATTATTATGAG

          *       1880          *       1900          *       1920
GAAGTGGTCAATGCTCAAGCGCAAGCTCAGGCTAGGGAGCAAGTAACAAAACAACCCTTC
GAAGTGGTCAATGCTCAAGCGCAAGCTCAGGCTAGGGAGCAAGTAACAAAACAACCCTTC

          *       1940          *       1960          *       1980
ACGATACAAGAGGAGACAGCAAAGTCAAGAGAAGGGAACTGCAGGCTCTTTGGCATTCCT
ACGATACAAGAGGAGACAGCAAAGTCAAGAGAAGGGAACTGCAGGCTCTTTGGCATTCCT

          *       2000          *       2020          *       2040
CTGACCAACAACATGAATGGGACAGACTCAACCATGTCTCAGAGAAACAACTTGAATGAT
CTGACCAACAACATGAATGGGACAGACTCAACCATGTCTCAGAGAAACAACTTGAATGAT

          *       2060          *       2080          *       2100
GCTGCGGGGCTTACACAGATAGCATCACCAAAGGTTCAGGACCTTTCAGATCAGTCAAAA
GCTGCGGGGCTTACACAGATAGCATCACCAAAGGTTCAGGACCTTTCAGATCAGTCAAAA

          *       2120          *       2140          *       2160
GGGTCAAAAATCAACAAACGATCATCGTGAACAGGGAAGACCATTCCAGACTAATAATCCT
GGGTCAAAAATCAACAAACGATCATCGTGAACAGGGAAGACCATTCCAGACTAATAATCCT

          *       2180          *       2200          *       2220
CATCCGAAGGATGCTCAAACGAAAACCAACTCAAGTAGGAGTTGCACAAAGGTTCACAAG
CATCCGAAGGATGCTCAAACGAAAACCAACTCAAGTAGGAGTTGCACAAAGGTTCACAAG

          *       2240          *       2260          *       2280
CAGGGAATTGCACTTGGCCGTTCAGTGGATCTTTCAAAGTTCCAAAACTATGAGGAGTTA
CAGGGAATTGCACTTGGCCGTTCAGTGGATCTTTCAAAGTTCCAAAACTATGAGGAGTTA

          *       2300          *       2320          *       2340
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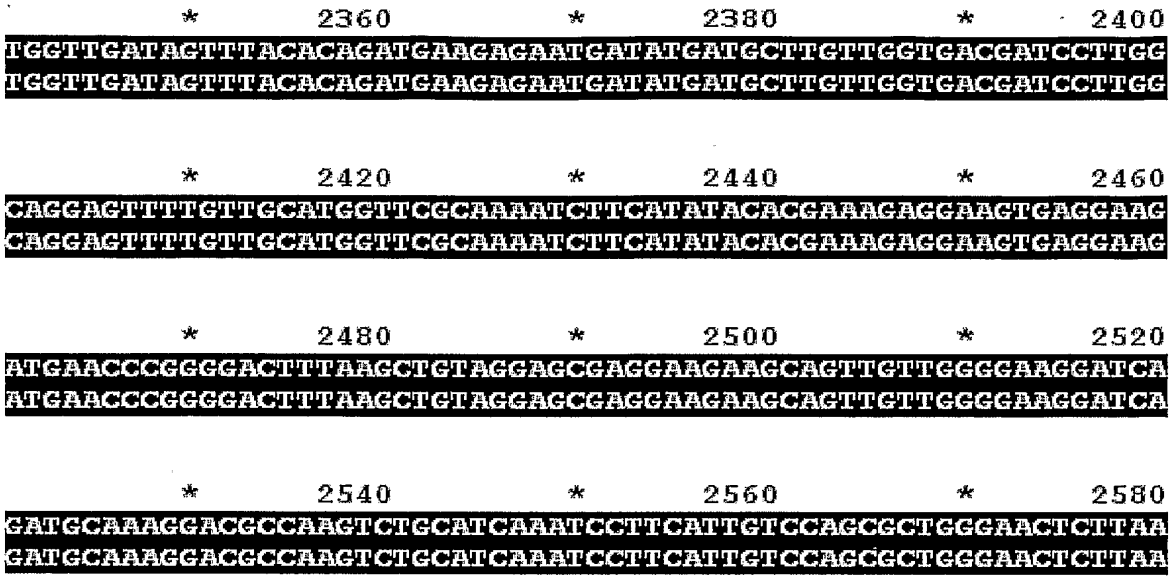
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TGGTTGATAGTTTACACAGATGAAGAGAATGATATGATGCTTGTTGGTGACGATCCTTGG
* 2420 * 2440 * 2460
CAGGAGTTTTGTTGCATGGTTCGCAAAATCTTCATATACACGAAAGAGGAAGTGAGGAAG
CAGGAGTTTTGTTGCATGGTTCGCAAAATCTTCATATACACGAAAGAGGAAGTGAGGAAG
* 2480 * 2500 * 2520
ATGAACCCGGGGACTTTAAGCTGTAGGAGCGAGGAAGAAGCAGTTGTTGGGGAAGGATCA
ATGAACCCGGGGACTTTAAGCTGTAGGAGCGAGGAAGAAGCAGTTGTTGGGGAAGGATCA
* 2540 * 2560 * 2580
GATGCAAAGGACGCCAAGTCTGCATCAAATCCTTCATTGTCCAGCGCTGGGAACTCTTAA
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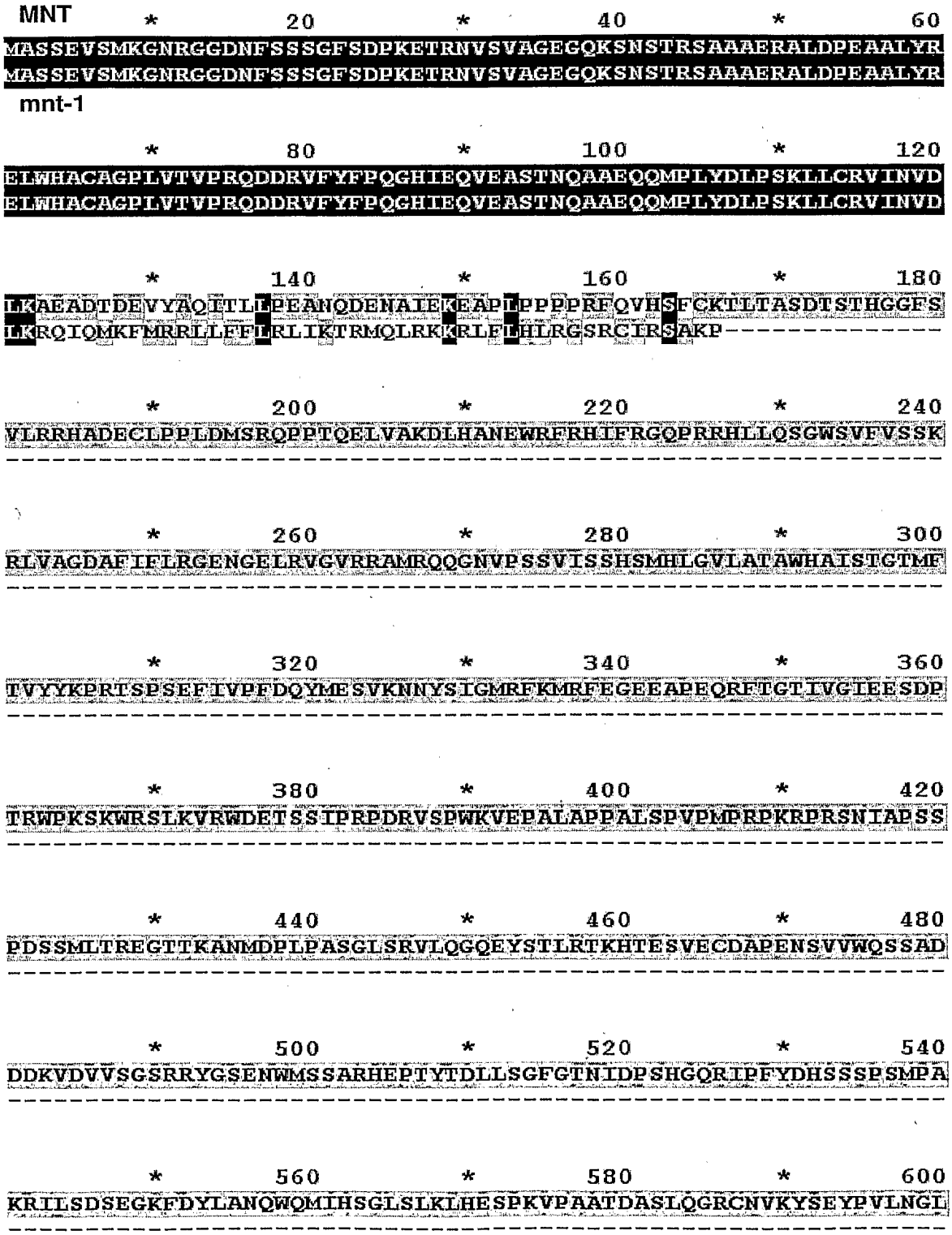
Figure 7

Alignment of w.t. MNT and mutant mnt-1 protein



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STENAGGNWPIRPRALNYYEEVVNAQAQAQAREQVIKQPFTIQEETAKSREGNCRLFGIP
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          *         680         *         700         *         720
LTNNMNGTDSTMSQRNNLNDAAGLTQIASPKVQDLSDQSKGSKSTNDHREQGRPFQTNNP
------------------------------------------------------------

          *         740         *         760         *         780
HPKDAQTKTNSSRSCTKVHKQGIALGRSVDLSKFQNYEELVAELDRLFEFNGELMAPKKD
------------------------------------------------------------

          *         800         *         820         *         840
WLIVYIDEENDMMLVGDDPWQEFCCMVRKIFIYTKEEVRKMNPGILSCRSEEEAVVGEGS
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          *
DAKDAKSASNPSLSSAGNS
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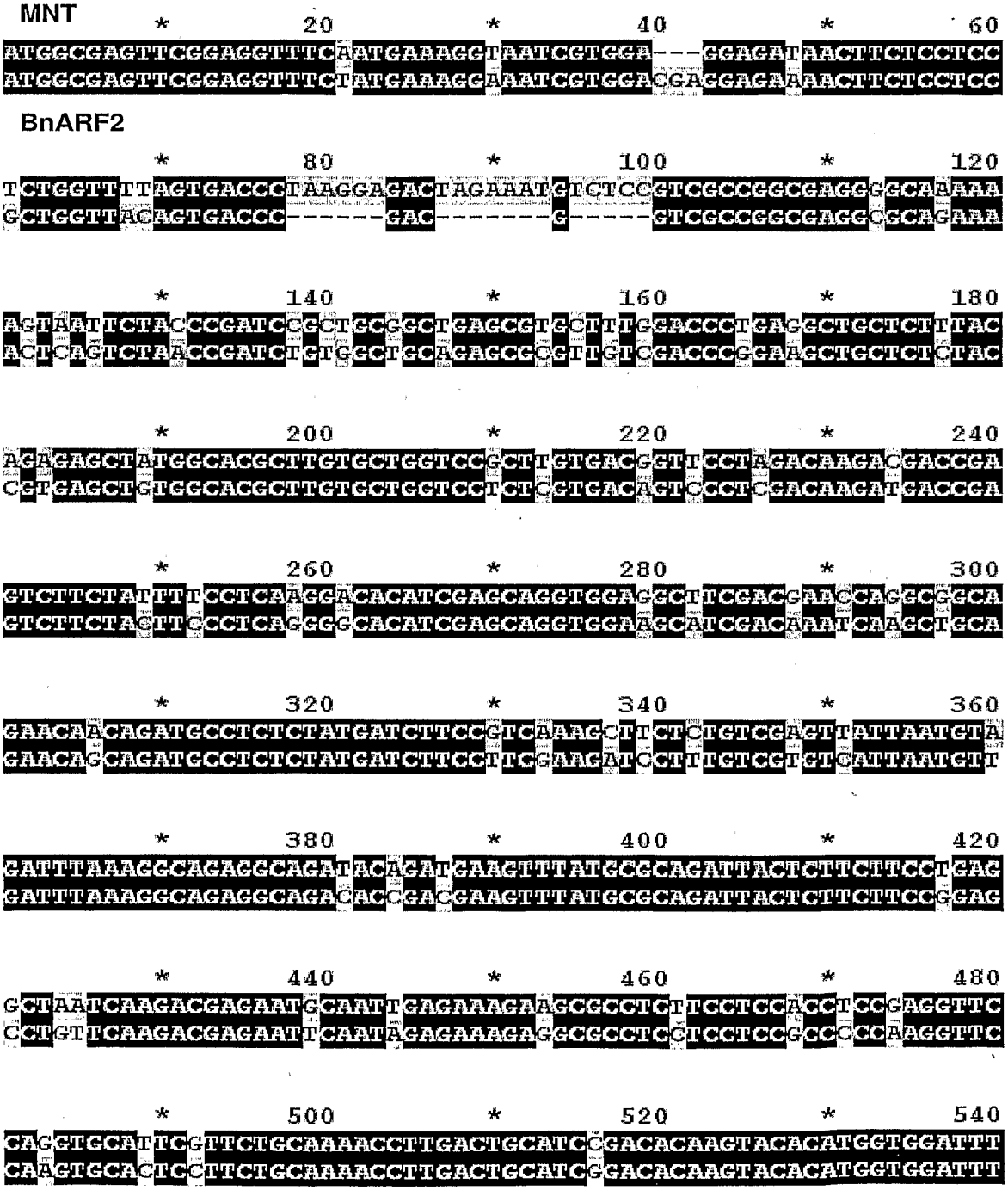
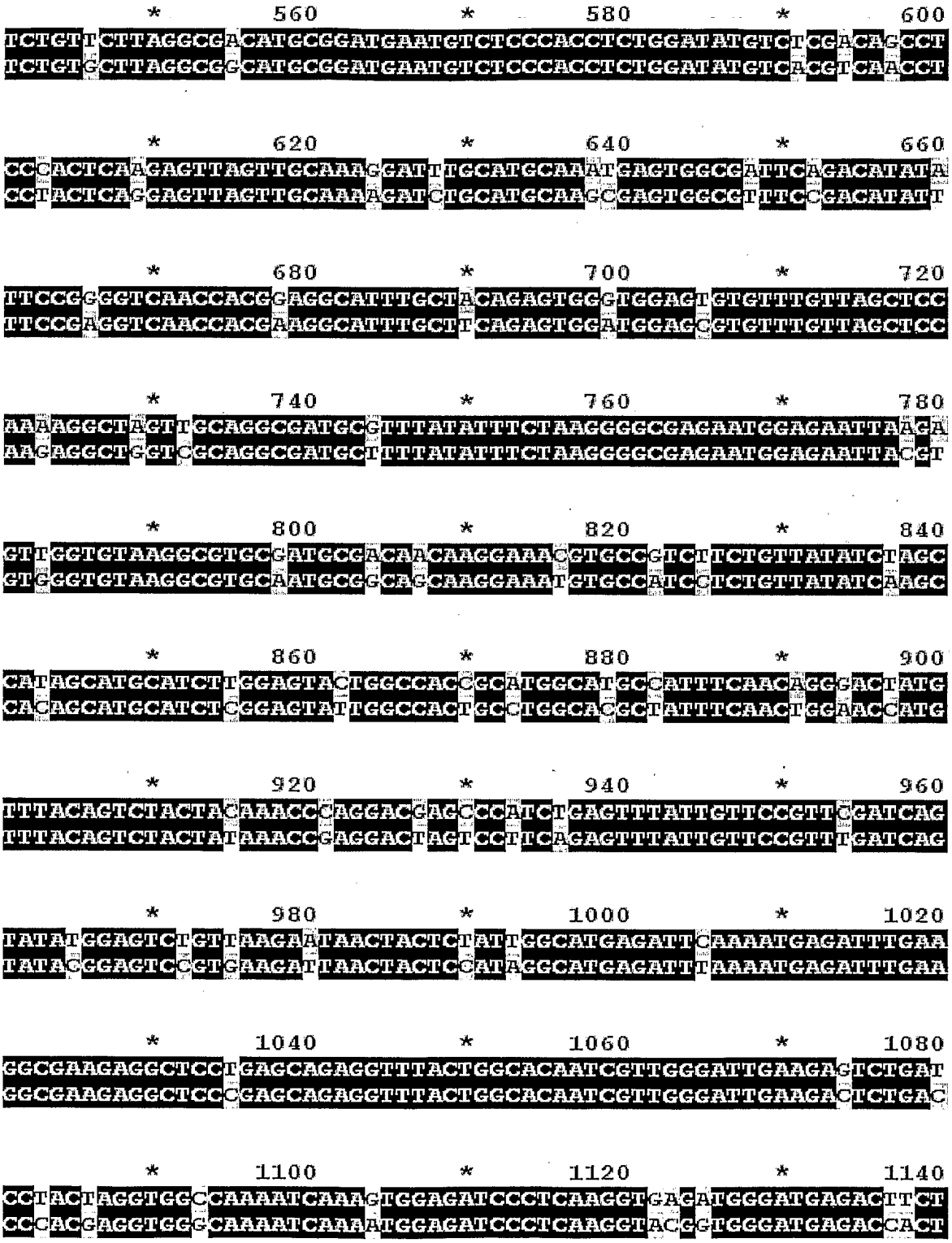
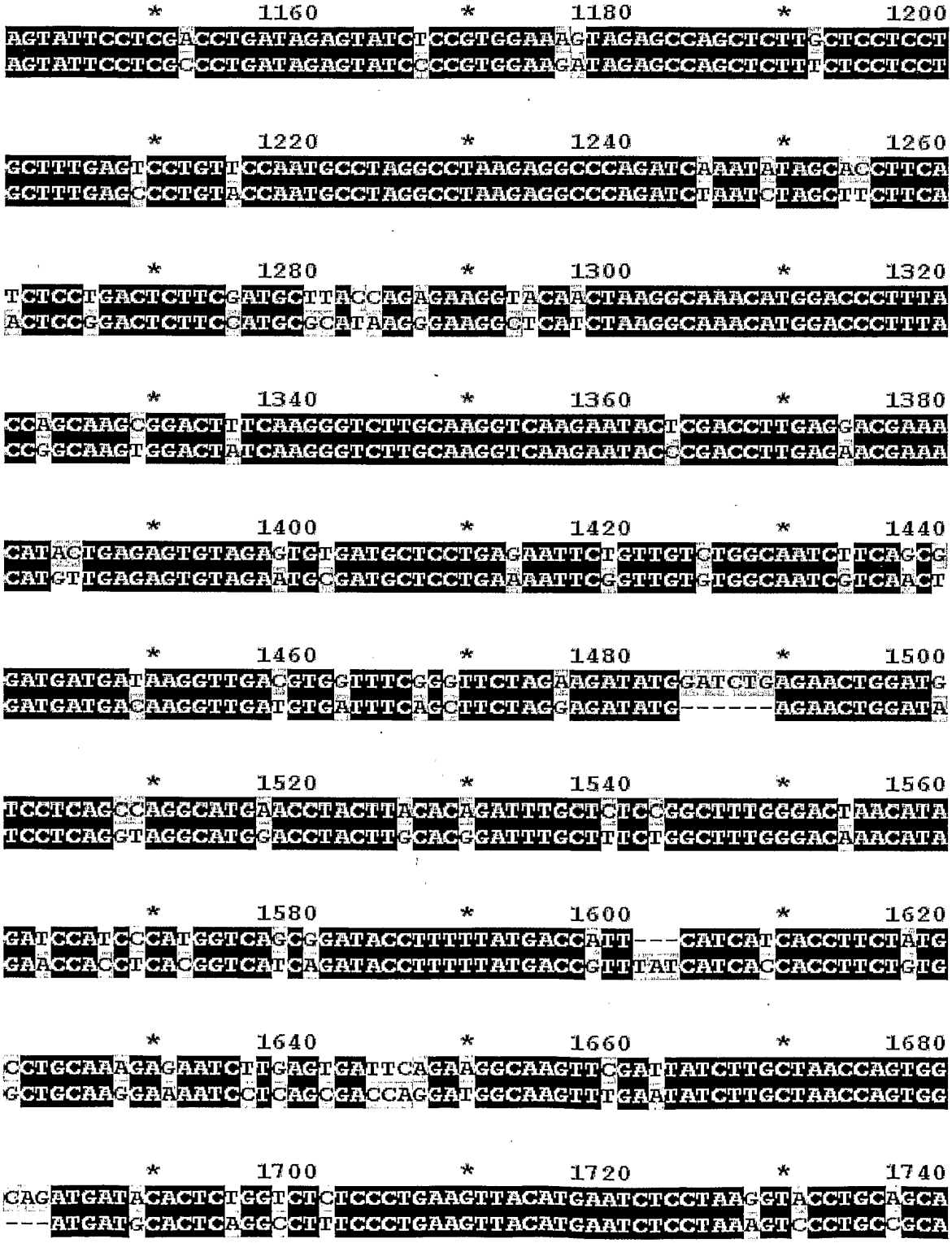
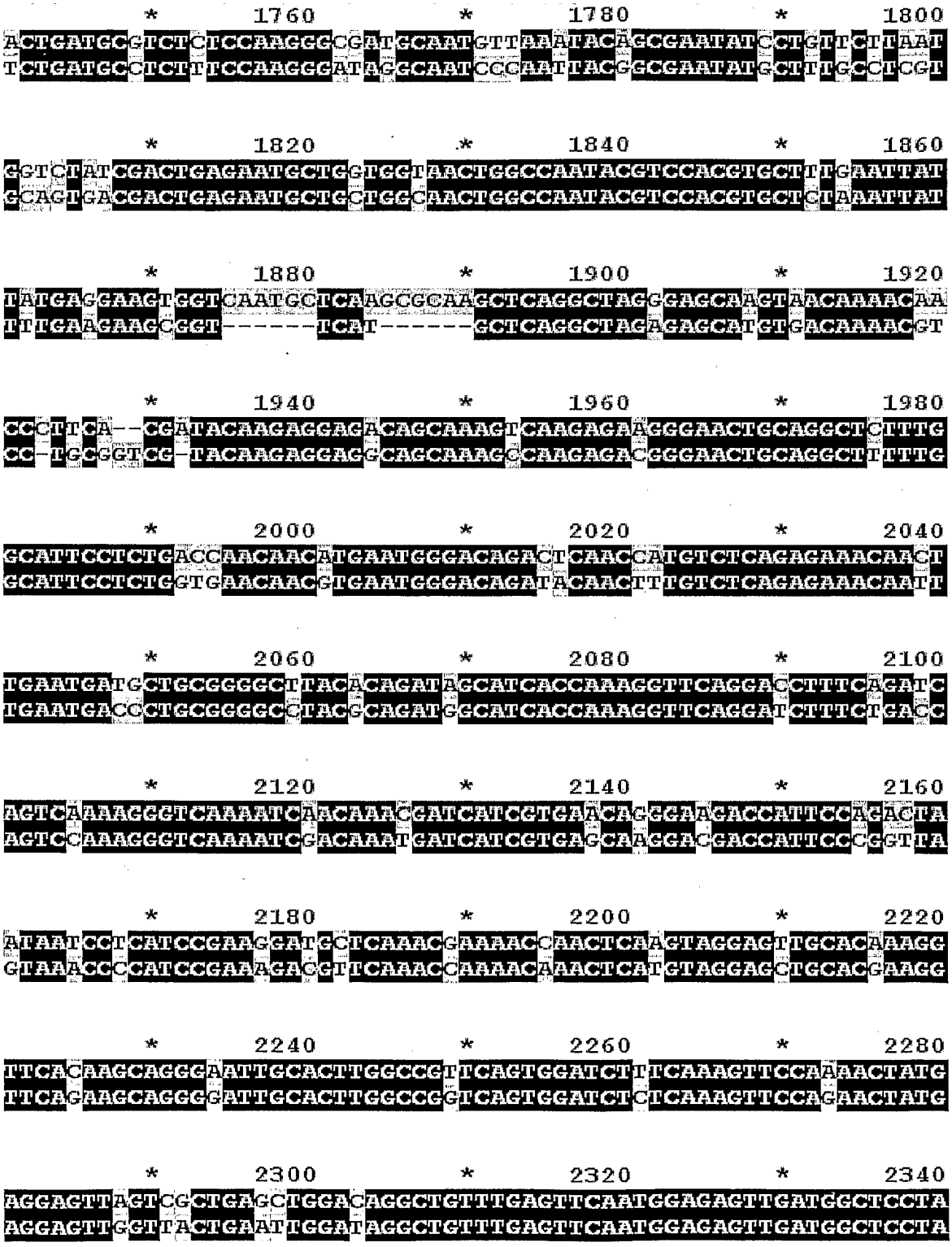
Figure 8

Alignment of MNT and BnARF2 cDNA




560
580
600
620
640
660
680
700
720
740
760
780
800
820
840
860
880
900
920
940
960
980
1000
1020
1040
1060
1080
1100
1120
1140


* 1160 * 1180 * 1200
AGTATTCCTCGACCTGATAGAGTATCTCCGTGGAAAGTAGAGCCAGCTCTTGCTCCTCCT
AGTATTCCTCGCCCTGATAGAGTATCCCCGTGGAAAGATAGAGCCAGCTCTTTCTCCTCCT
* 1220 * 1240 * 1260
GCTTTGAGTCCTGTTCCAATGCCTAGGCCTAAGAGGCCCAGATCAAATATAGCACCTTCA
GCTTTGAGCCCTGTACCAATGCCTAGGCCTAAGAGGCCCCAGATCTAATCTAGCTTCTTCA
* 1280 * 1300 * 1320
TCTCCTGACTCTTCGATGCTTACCAGAGAAGGTACAACTAAGGCAAACATGGACCCTTTA
ACTCCGGACTCTTCCATGCGCATAAGGGAAGGCTCATCTAAGGCAAACATGGACCCTTTA
* 1340 * 1360 * 1380
CCAGCAAGCGGACTTTCAAGGGTCTTGCAAGGTCAACAATACTCGACCTTGAGGACGAAA
CCGGCAAGTGGACTATCAAGGGTCTTGCAAGGTCAACAATACCCGACCTTGAGAACGAAA
* 1400 * 1420 * 1440
CATACTGAGAGTGTAGAGTGTGATGCTCCTGAGAATTCTGTTGTCTGGCAATCTTCAGCG
CATGTTGAGAGTGTAGAATGCGATGCTCCTGAAAATTCGGTTGTGTGGCAATCGTCAACT
* 1460 * 1480 * 1500
GATGATGATAAGGTTGACGTGGTTTCGGGTTCTAGAAGATATGGATCTGAGAACTGGATG
GATGATGACAAGGTTGATGTGATTTCAGCTTCTAGGAGATATG------AGAACTGGATA
* 1520 * 1540 * 1560
TCCTCAGCCAGGCATGAACCTACTTACACAGATTTGCTCTCCGGCTTTGGGACTAACATA
TCCTCAGGTAGGCATGGACCTACTTGCACGGATTTGCTTTCTGGCTTTGGGACAAACATA
* 1580 * 1600 * 1620
GATCCATCCCATGGTCAGCGGATACCTTTTTATGACCATT---CATCATCACCTTCTATG
GAACCACCTCACGGTCATCAGATACCTTTTTATGACCGTTTATCATCACCACCTTCTGTG
* 1640 * 1660 * 1680
CCTGCAAAGAGAATCTTGAGTGATTCAGAAGGCAAGTTCGATTATCTTGCTAACCAGTGG
GCTGCAAGGAAAATCCTCAGCGACCAGGATGGCAAGTTTGAATATCTTGCTAACCAGTGG
* 1700 * 1720 * 1740
CAGATGATACACTCTGGTCTCTCCCTGAAGTTACATGAATCTCCTAAGGTACCTGCAGCA
---ATGATGCACTCAGGCCTTTCCCTGAAGTTACATGAATCTCCTAAAGTCCCTGCCGCA


1760
1780
1800
1820
1840
1860
1880
1900
1920
1940
1960
1980
2000
2020
2040
2060
2080
2100
2120
2140
2160
2180
2200
2220
2240
2260
2280
2300
2320
2340

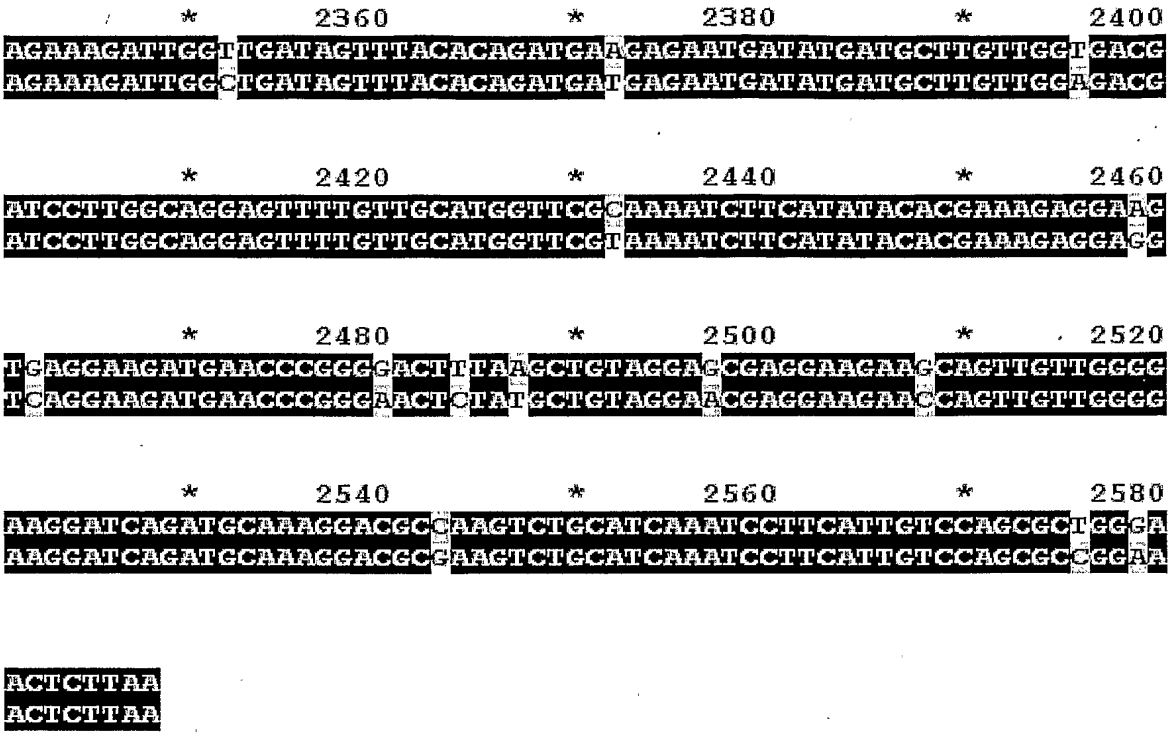
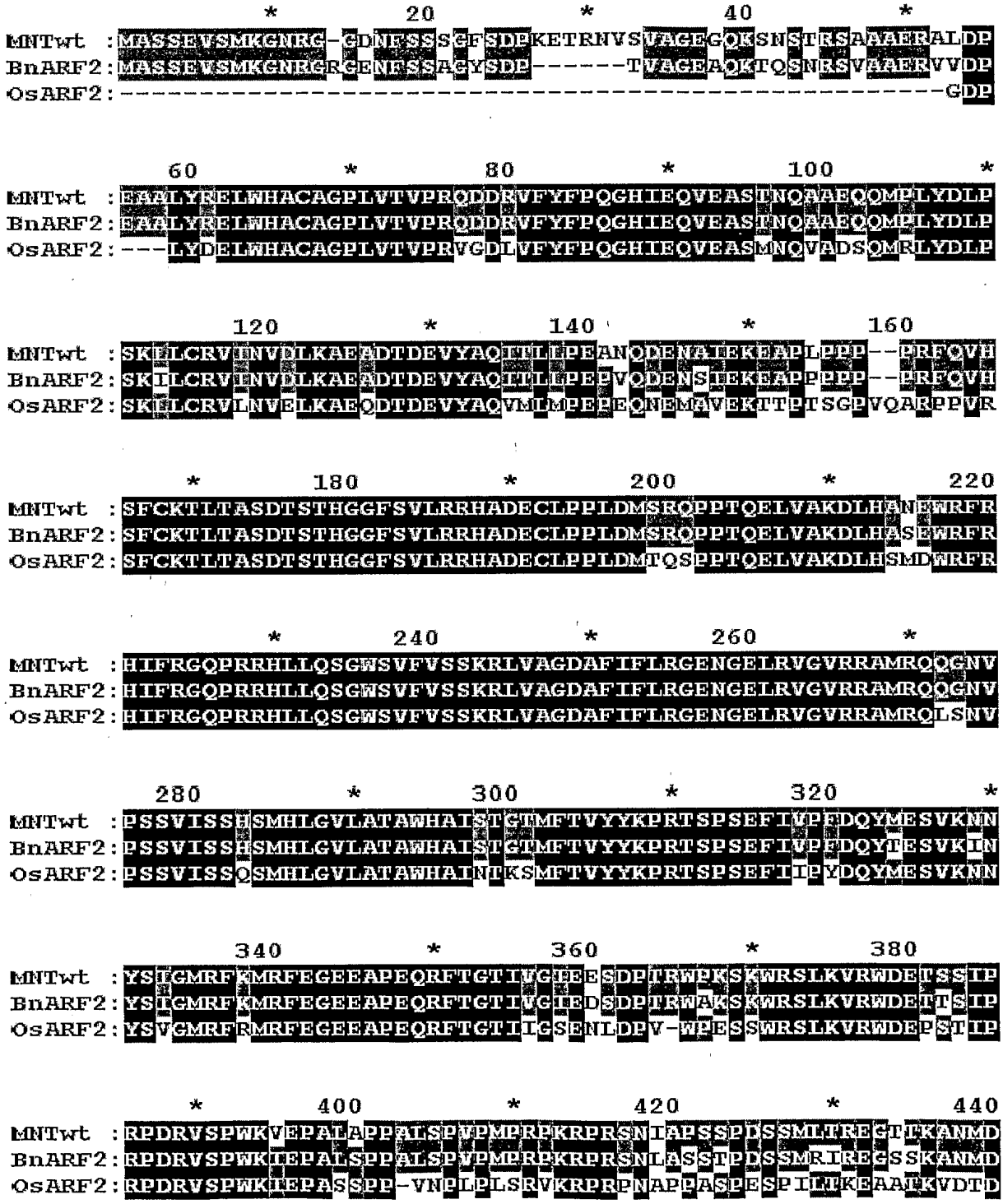

2360
2380
2400
AGAAAGATTGGTTGATAGTTTACACAGATGAAGAGAATGATATGATGCTTGTTGGTGACG
AGAAAGATTGGCTGATAGTTTACACAGATGATGAGAATGATATGATGCTTGTTGGAGACG
2420
2440
2460
ATCCTTGGCAGGAGTTTTGTTGCATGGTTCGCAAAATCTTCATATACACGAAAGAGGAAG
ATCCTTGGCAGGAGTTTTGTTGCATGGTTCGTAAAATCTTCATATACACGAAAGAGGAGG
2480
2500
2520
TGAGGAAGATGAACCCGGGGACTTTAAGCTGTAGGAGCGAGGAAGAAGCAGTTGTTGGGG
TCAGGAAGATGAACCCGGGAACTCTATGCTGTAGGAACGAGGAAGAACCAGTTGTTGGGG
2540
2560
2580
AAGGATCAGATGCAAAGGACGCCAAGTCTGCATCAAATCCTTCATTGTCCAGCGCTGGGA
AAGGATCAGATGCAAAGGACGCGAAGTCTGCATCAAATCCTTCATTGTCCAGCGCCGGAA
ACTCTTAA
ACTCTTAA

Figure 9

Alignment of MNT, BnARF2, OsARF2 proteins



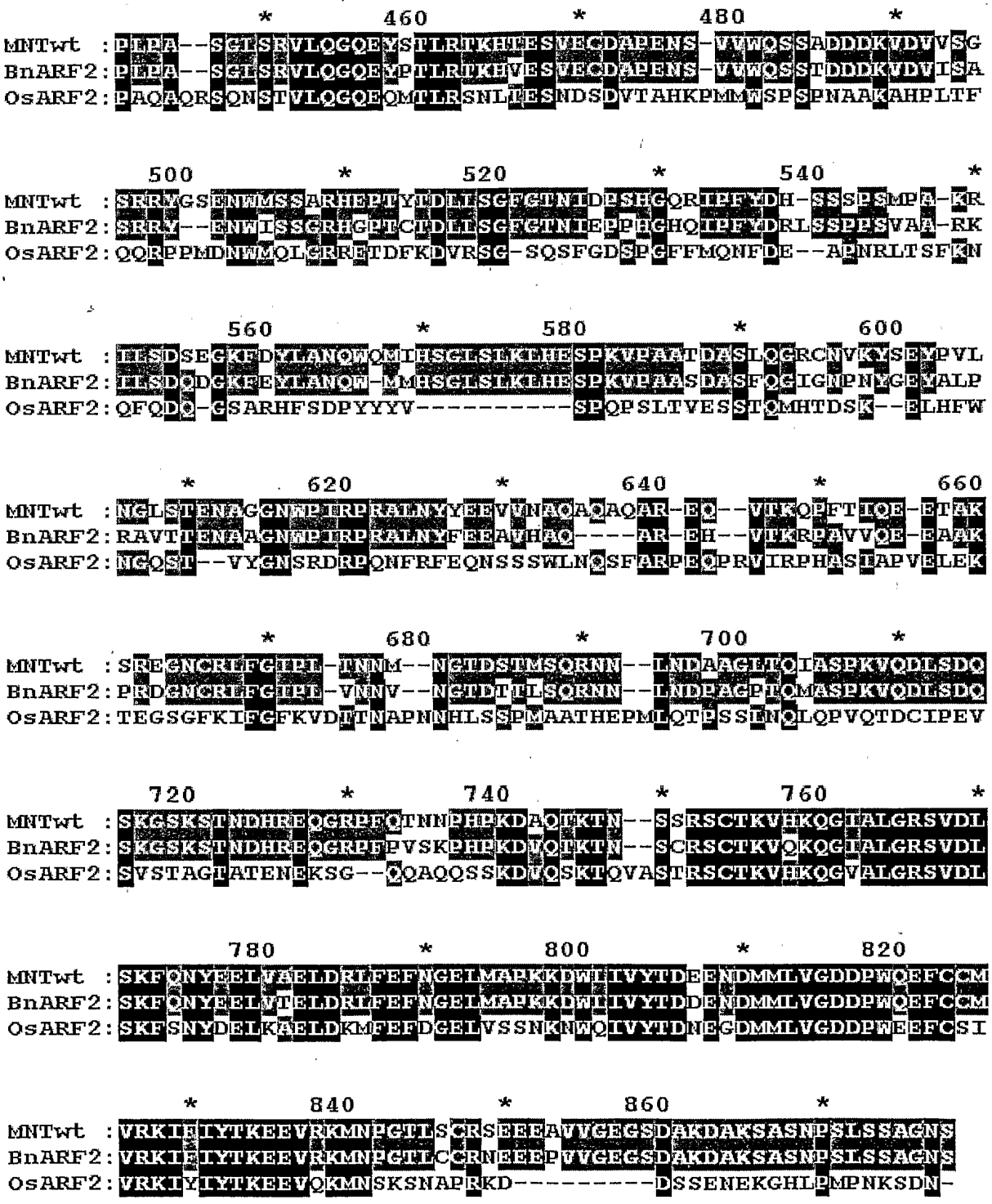
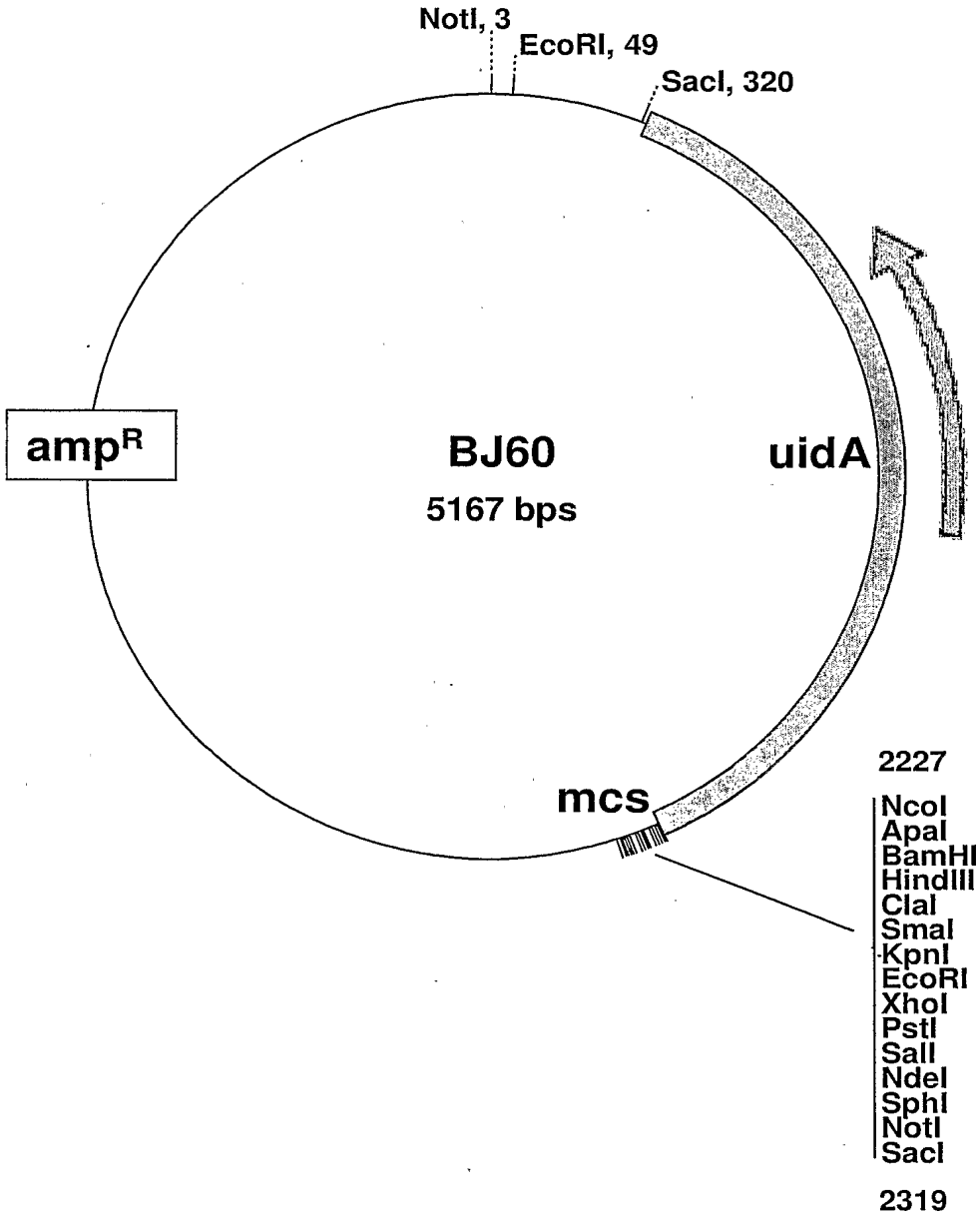
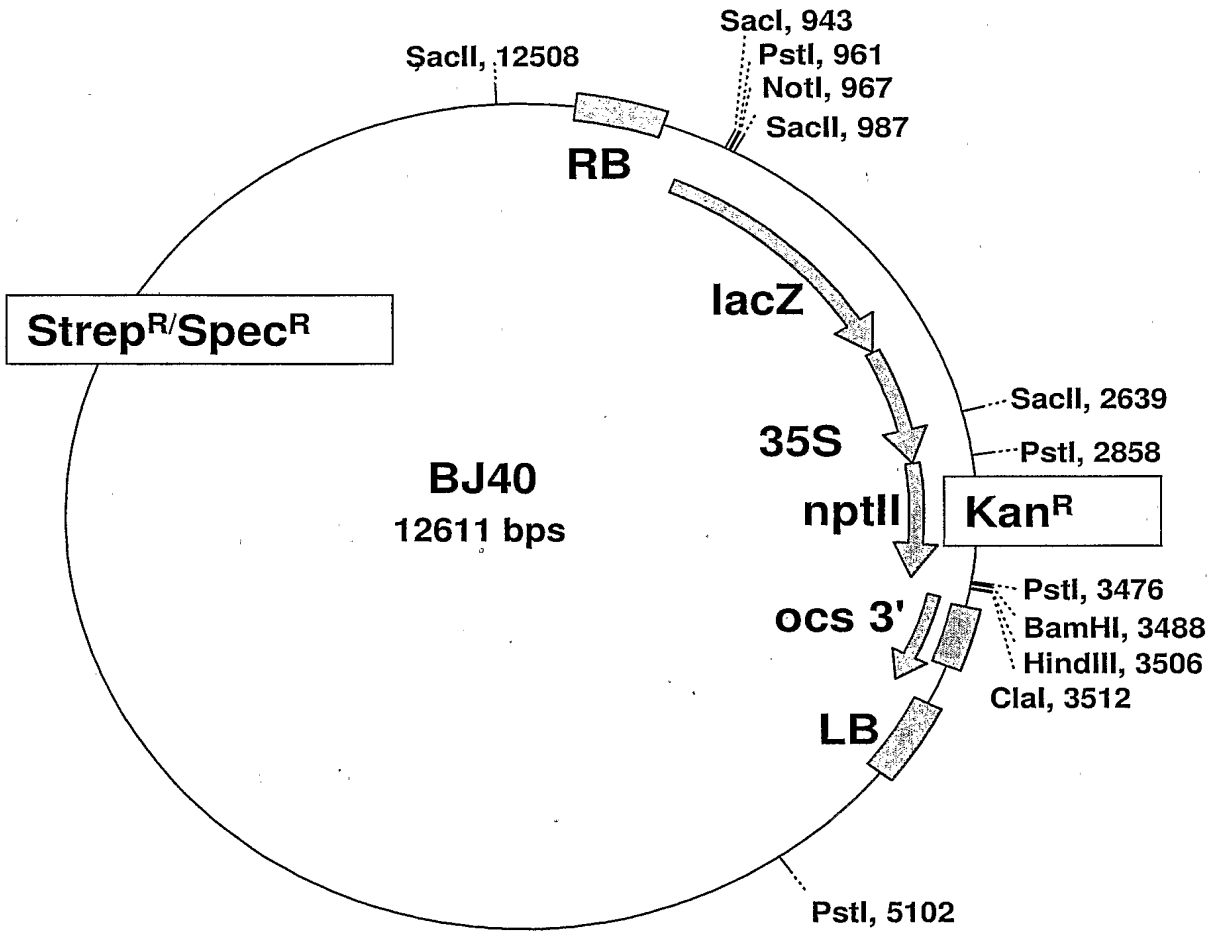

460
480
MNTwt : PLLA--SGLSRVLQGQEYSTLRTKHTESVECDAPENS-VVWQSSADDDKVDVVSG
BnARF2: PLLA--SGLSRVLQGQEYPTLRTKHVESVECDAPENS-VVWQSSTDDDKVDVISA
OsARF2: PAQAQRSQNSTVLQGQEQMTLRSNLTESNDSDVTAHKPMMWSPSPNAAKAHPLTF
500
520
540
MNTwt : SRRYGSENWMSSARHEPTYTDLLISGFGTNIDPSHGQRIPFYDH-SSSPSMPA-KR
BnARF2: SRRY--ENWISSGRHGPTCTDLLISGFGTNIEPPHGHQIPFYDRLSSPPSVAA-RK
OsARF2: QQRPPMDNWMQLGRRETDFKDVRSG-SQSFGDSPGFFMQNFDE--APNRLTSFKN
560
580
600
MNTwt : LLSDSEGKFDYLANQWQMIHSGLSLKLHESPKVPAATDASLQGRCNVKYSEYPVL
BnARF2: LLSDQDGKFEYLANQW-MMHSGLSLKLHESPKVPAASDASFQGIGNPNYGEYALP
OsARF2: QFQDQ-GSARHFSDPYYYV----------SPQPSLTVESSTQMHTDSK--ELHFW
620
640
660
MNTwt : NGLSTENAGGNWPIRPRALNYYEEVVNAQAQAQAR-EQ--VTKQPFTIQE-ETAK
BnARF2: RAVTTENAAGNWPIRPRALNYFEEAVHAQ----AR-EH--VTKRPAVVQE-EAAK
OsARF2: NGQST--VYGNSRDRPQNFRFEQNSSSWLNQSFARPEQPRVIRPHASIAPVELEK
680
700
MNTwt : SFEGNGRLFGIPL-INNM--NGTDSTMSQRNN--LNDAAGLTQIASPKVQDLSDQ
BnARF2: PRDGNGRLFGIPL-VNNV--NGTDITLSQRNN--LNDPAGPTQMASPKVQDLSDQ
OsARF2: TEGSGFKIFGFKVDLTNAPNNHLSSPMAATHEPMLQTPSSLNQLQPVQTDCIPEV
720
740
760
MNTwt : SKGSKSTNDHREQGRPFQTNNPHPKDAQTKTN--SSRSCTKVHKQGIALGRSVDL
BnARF2: SKGSKSTNDHREQGRPFPVSKPHPKDVQTKTN--SCRSCTKVQKQGIALGRSVDL
OsARF2: SVSTAGLATENEKSG--QQAQQSSKDVQSKTQVASTRSCTKVHKQGVALGRSVDL
780
800
820
MNTwt : SKFQNYEELVAELDRLFEFNGELMAPKKDWLIVYTDEENDMMLVGDDPWQEFCCM
BnARF2: SKFQNYEELVTELDRLFEFNGELMAPKKDWLIVYTDDENDMMLVGDDPWQEFCCM
OsARF2: SKFSNYDELKAELDKMFEFDGELVSSNKNWQIVYTDNEGDMMLVGDDPWEEFCSI
840
860
MNTwt : VRKLFIYTKEEVRKMNPGTLSCRSEEEAVVGEGSDAKDAKSASNPSLSSAGNS
BnARF2: VRKLFIYTKEEVRKMNPGTLCCRNEEEPVVGEGSDAKDAKSASNPSLSSAGNS
OsARF2: VRKIYIYTKEEVQKMNSKSNAPRKD----------DSSENEKGHLPMPNKSDN-

Figure 10
Vectors used for cloning

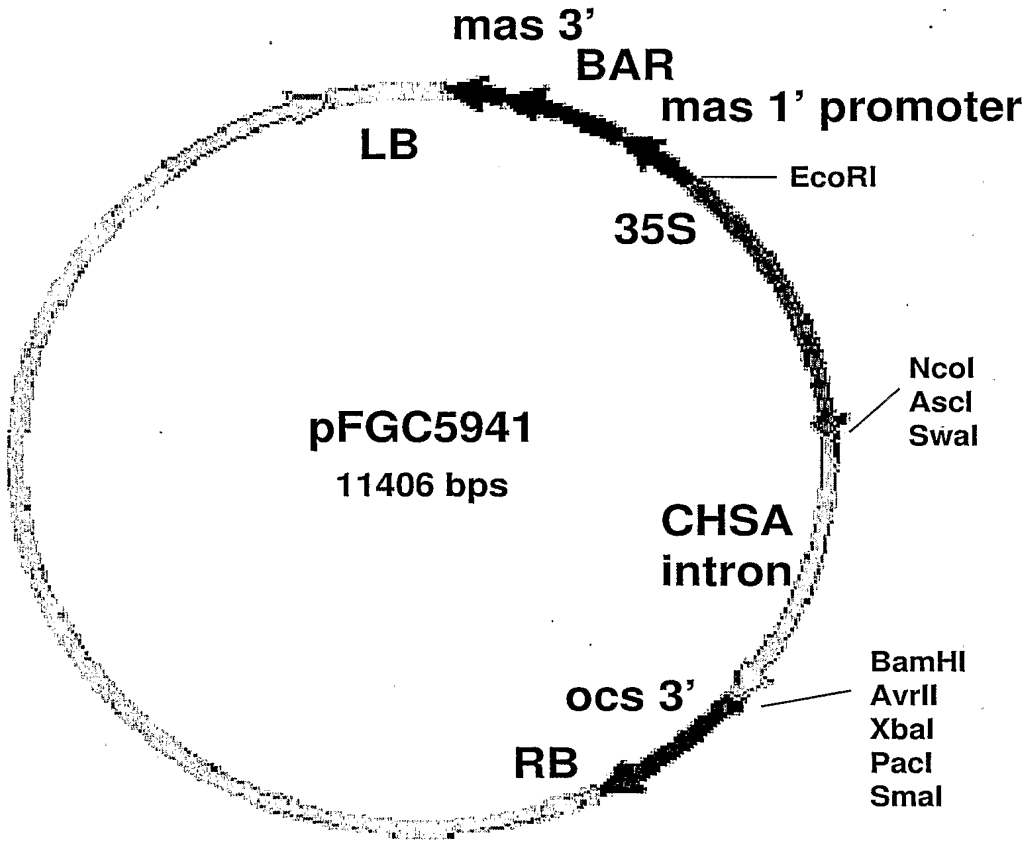
10A BJ60



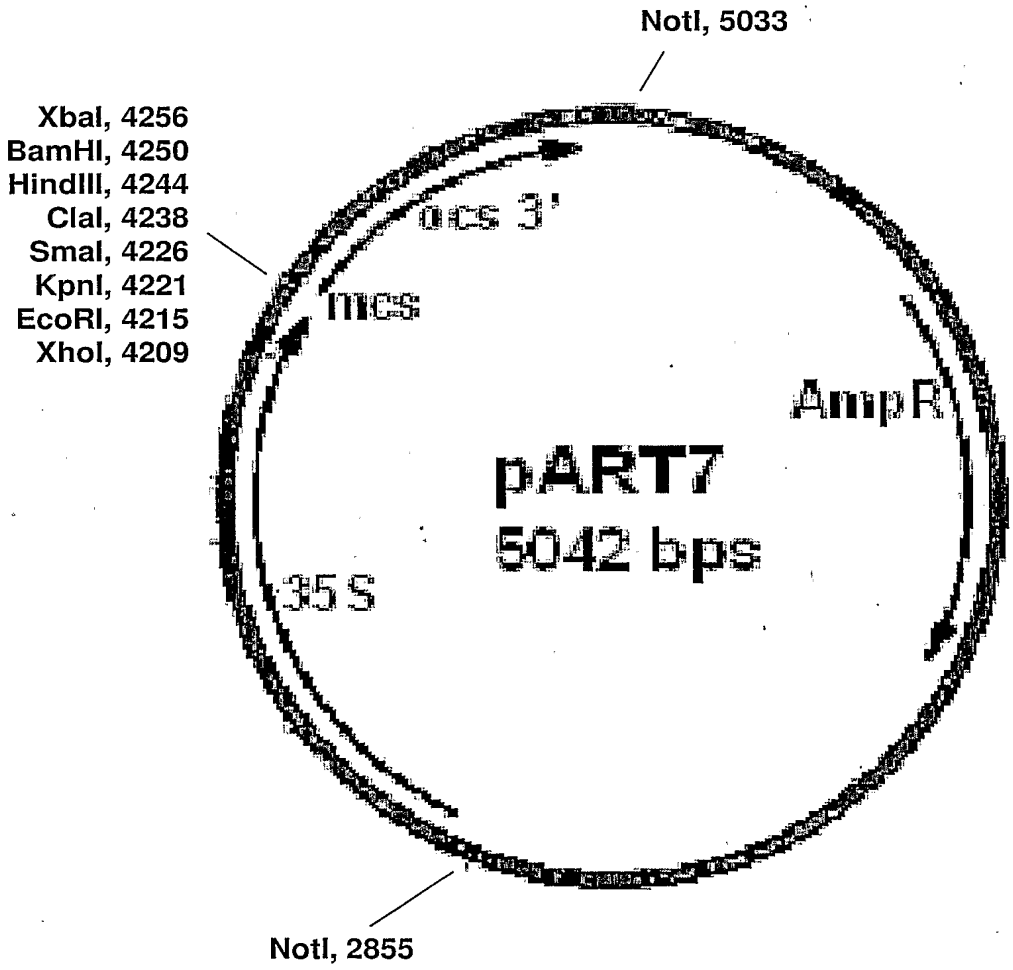
10B BJ40


SacII, 12508
SacI, 943
PstI, 961
NotI, 967
SacII, 987
RB
lacZ
Strep^R/Spec^R
35S
SacII, 2639
PstI, 2858
BJ40
12611 bps
nptII
Kan^R
PstI, 3476
BamHI, 3488
HindIII, 3506
ClaI, 3512
ocs 3'
LB
PstI, 5102

10C pFGC5941



10D pART7


NotI, 5033
XbaI, 4256
BamHI, 4250
HindIII, 4244
ClaI, 4238
SmaI, 4226
KpnI, 4221
EcoRI, 4215
XhoI, 4209
ocs 3'
mcs
AmpR
pART7
5042 bps
35 S
NotI, 2855

10E BJ36

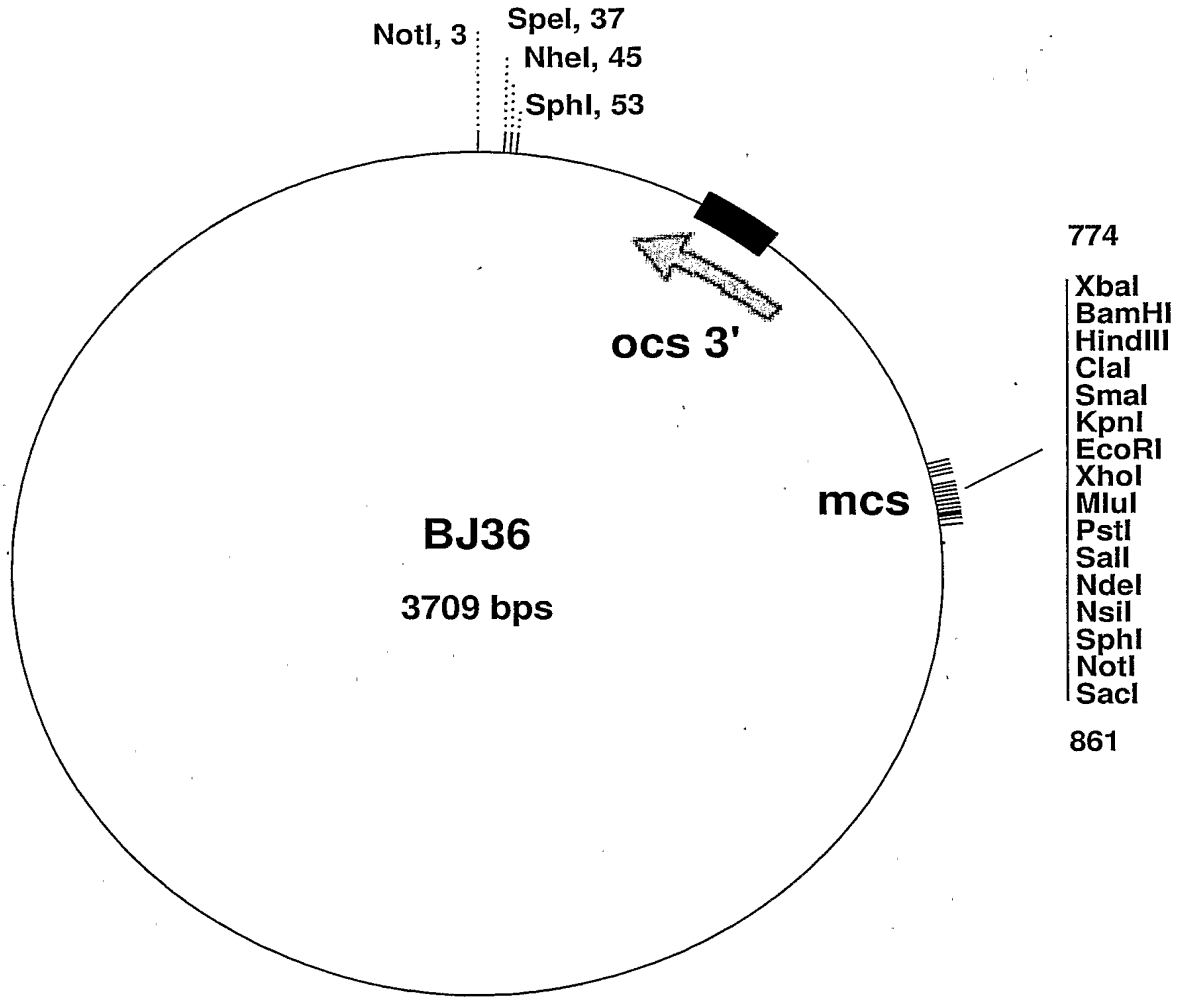
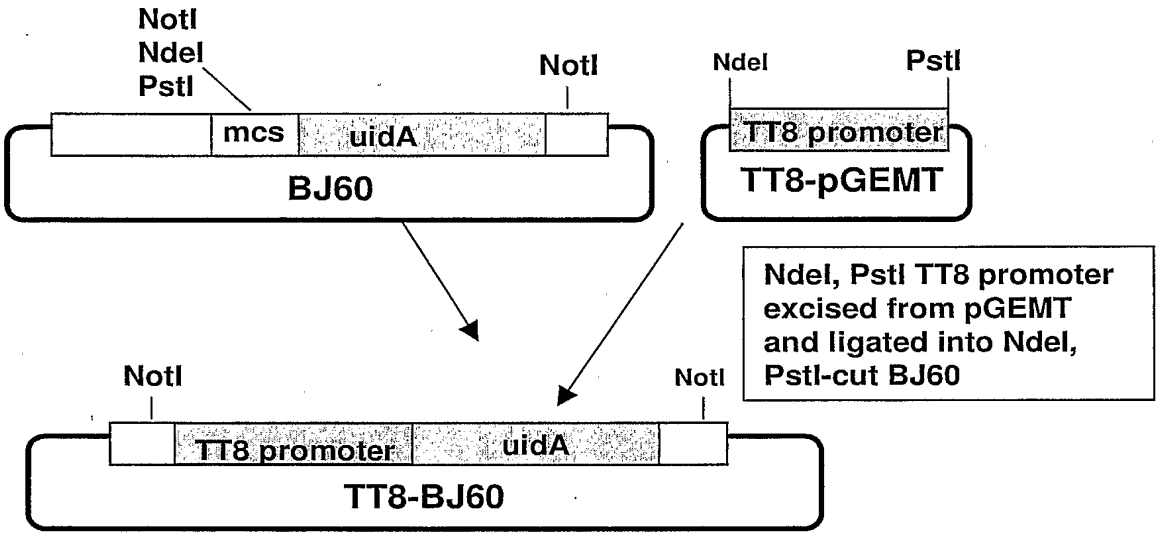

NotI, 3
SpeI, 37
NheI, 45
SphI, 53
ocs 3'
mcs
BJ36
3709 bps
774
XbaI
BamHI
HindIII
ClaI
SmaI
KpnI
EcoRI
XhoI
MluI
PstI
SalI
NdeI
NsiI
SphI
NotI
SacI
861

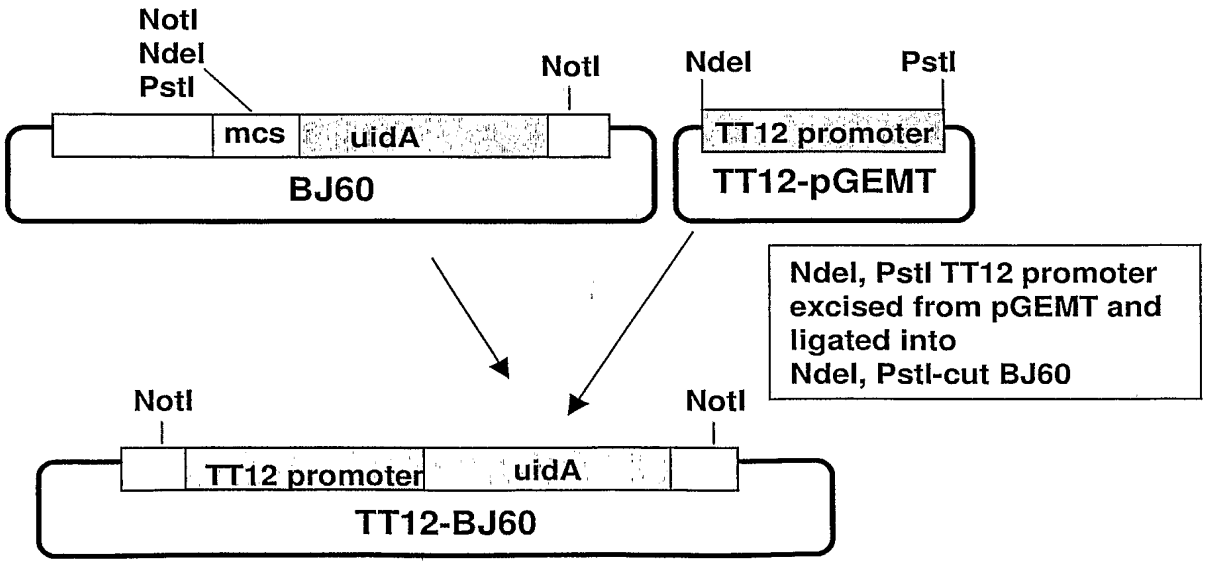
Figure 11

Cloning strategy, Example 3

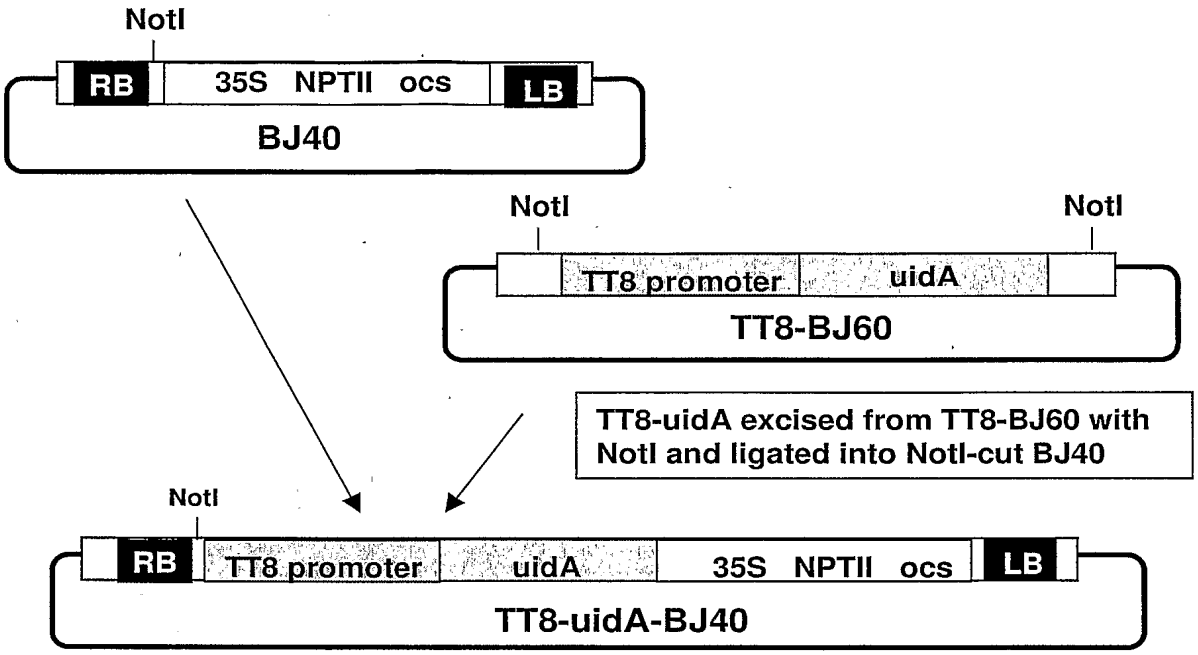
Example 3a(i)



Example 3a(ii)



Example 3b(i)



Example 3b(ii)

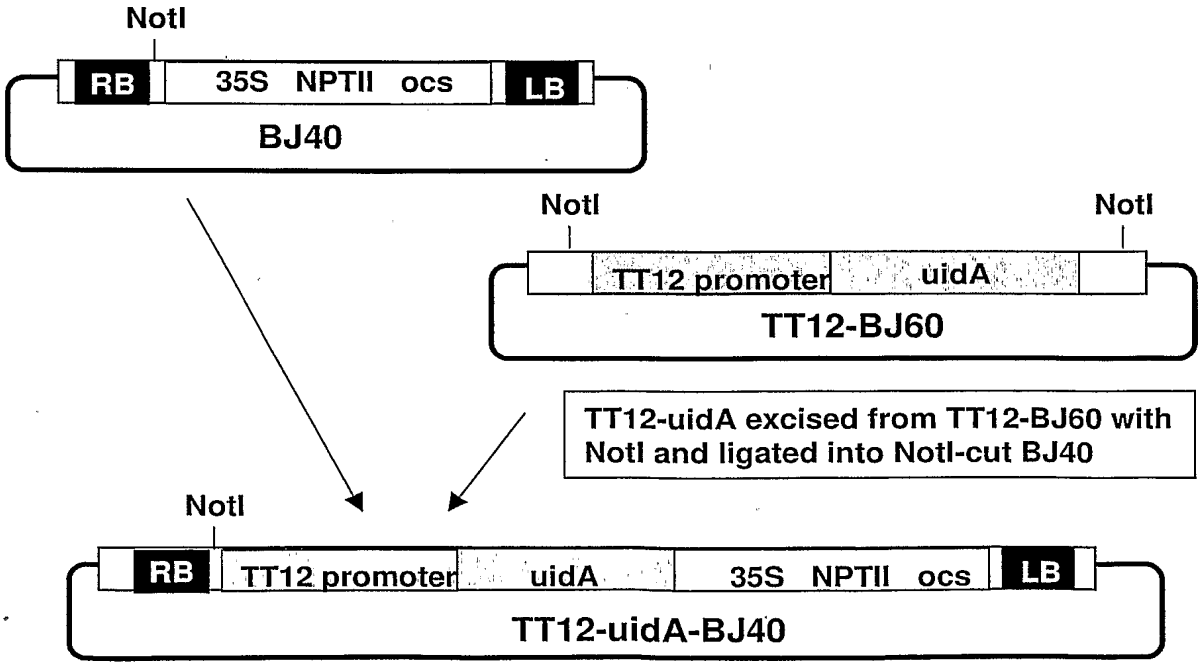


Figure12

TT12::GUS

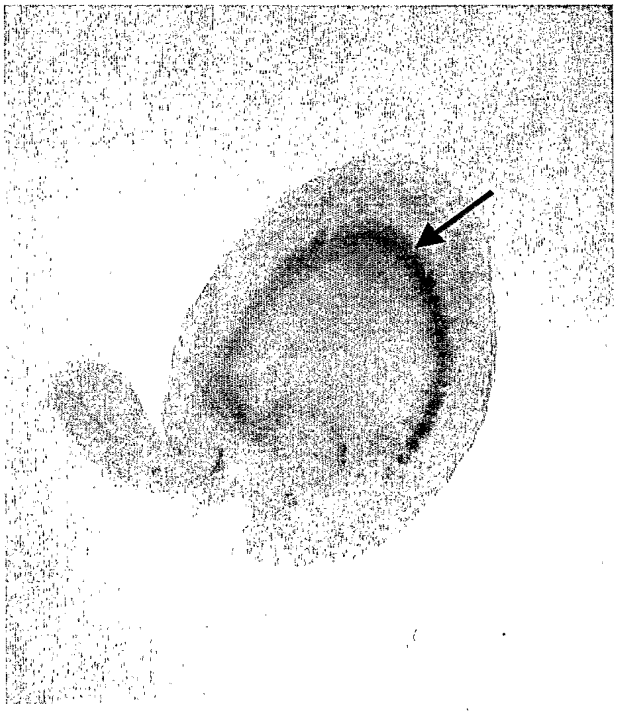


Figure 13
Cloning strategy, Example 4

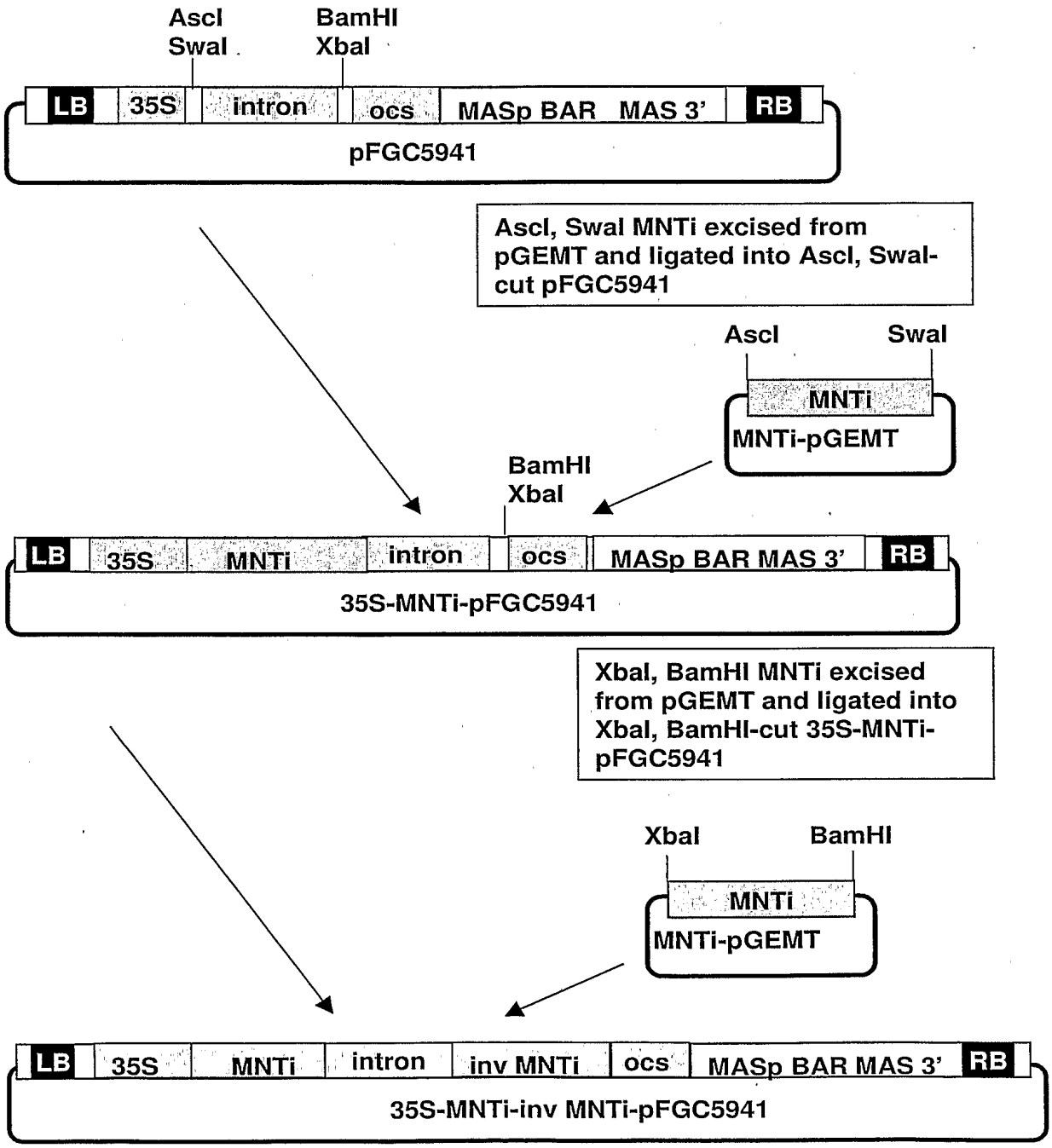


Figure 14
Cloning strategy, Example 5

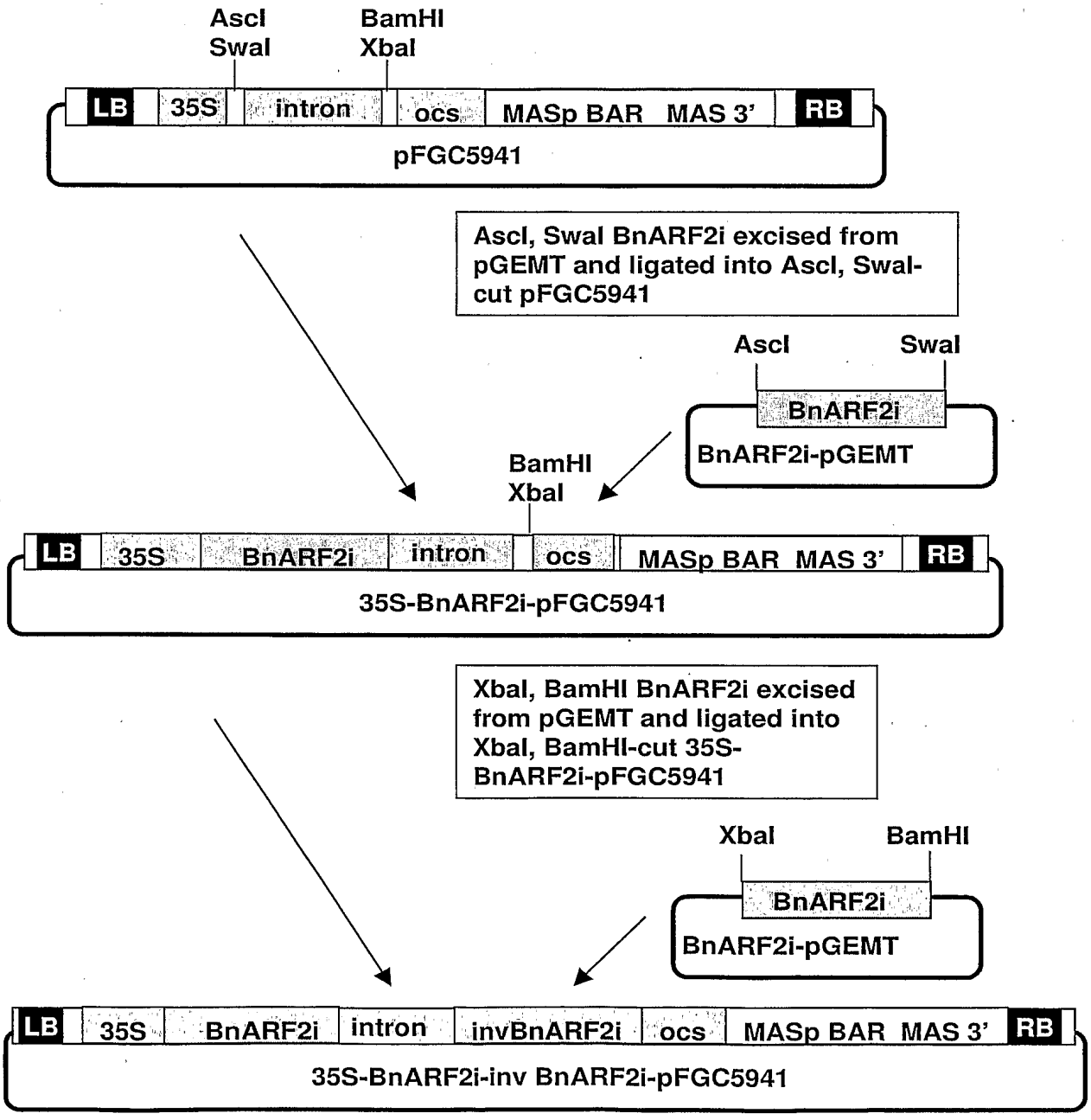
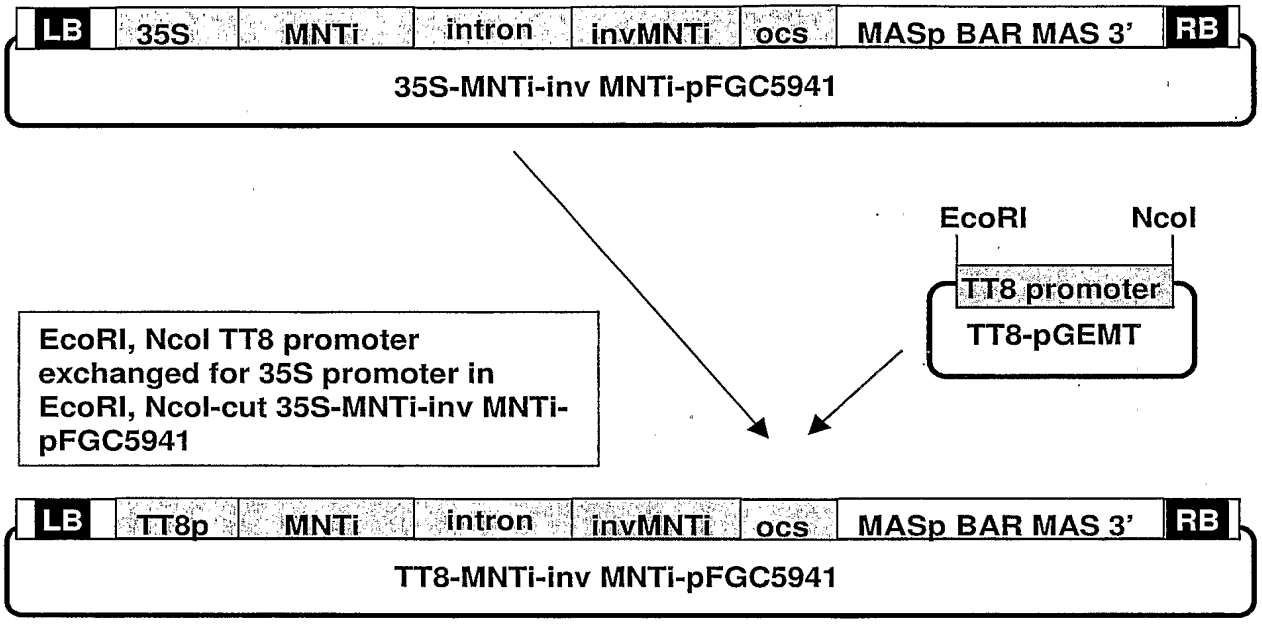


Figure 15
Cloning strategy, Example 6

Example 6a(i)



Example 6a(ii)

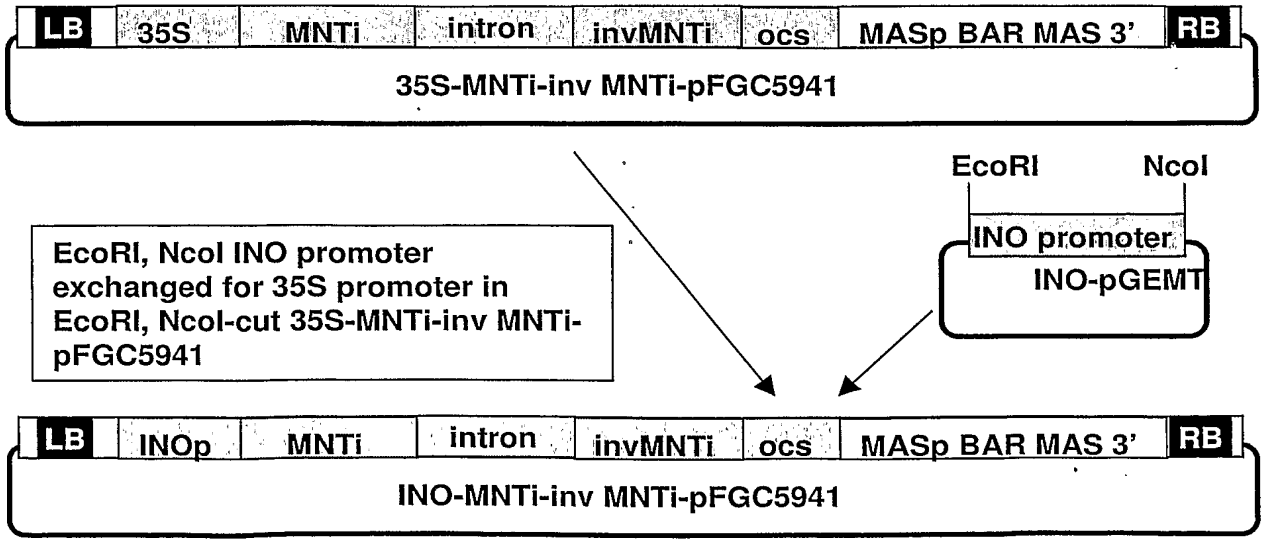
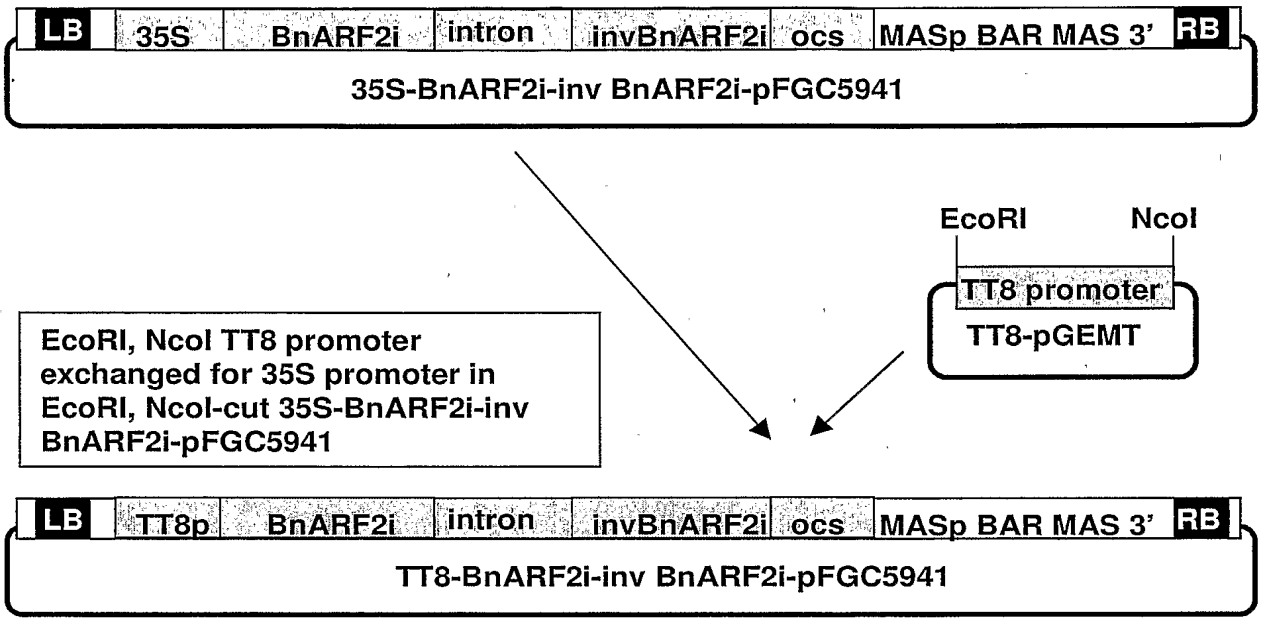


Figure 16
Cloning strategy, Example 7

Example 7a(i)



Example 7a(ii)

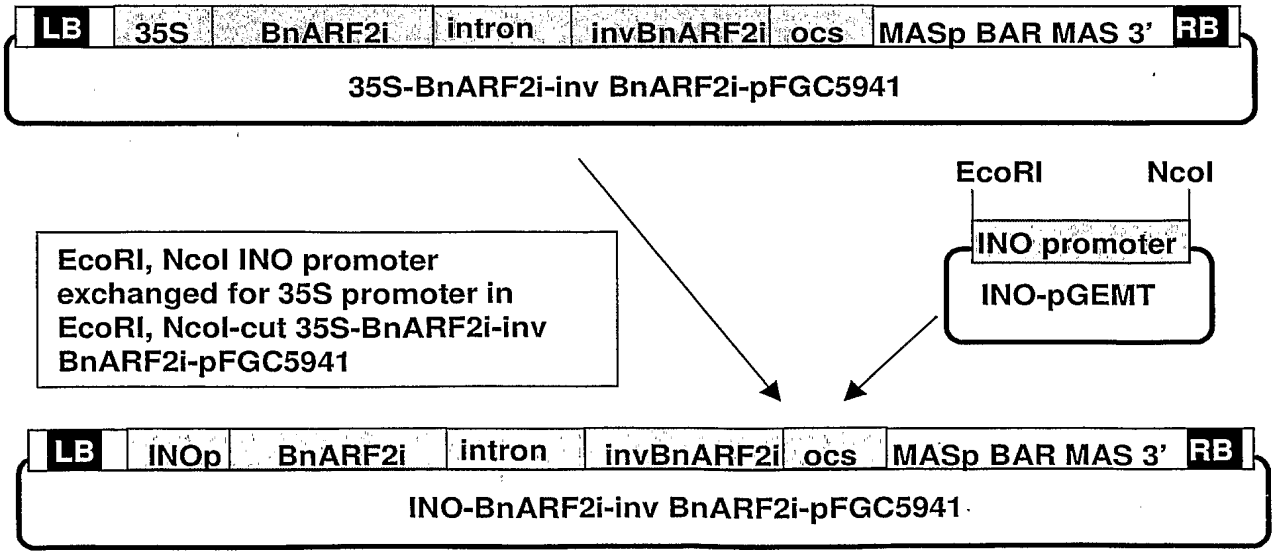
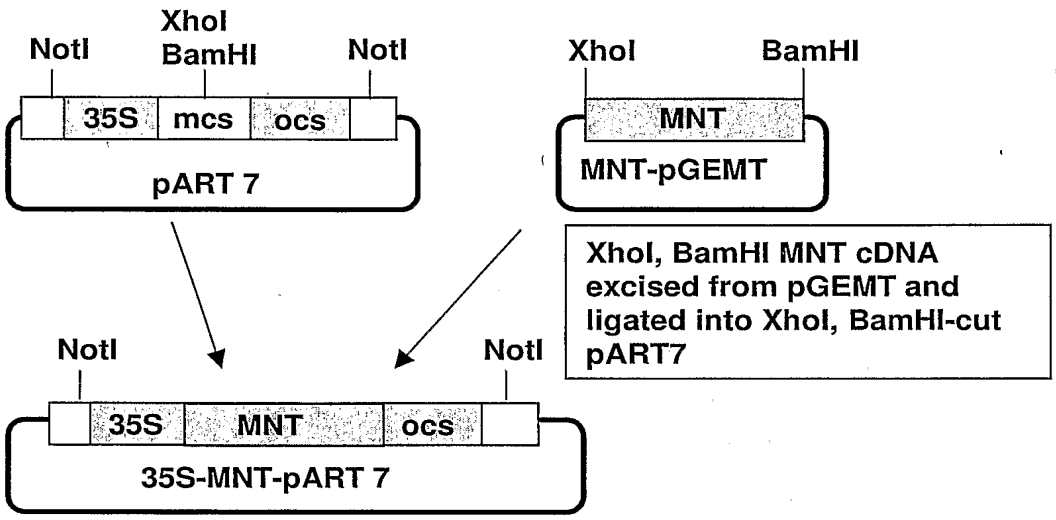
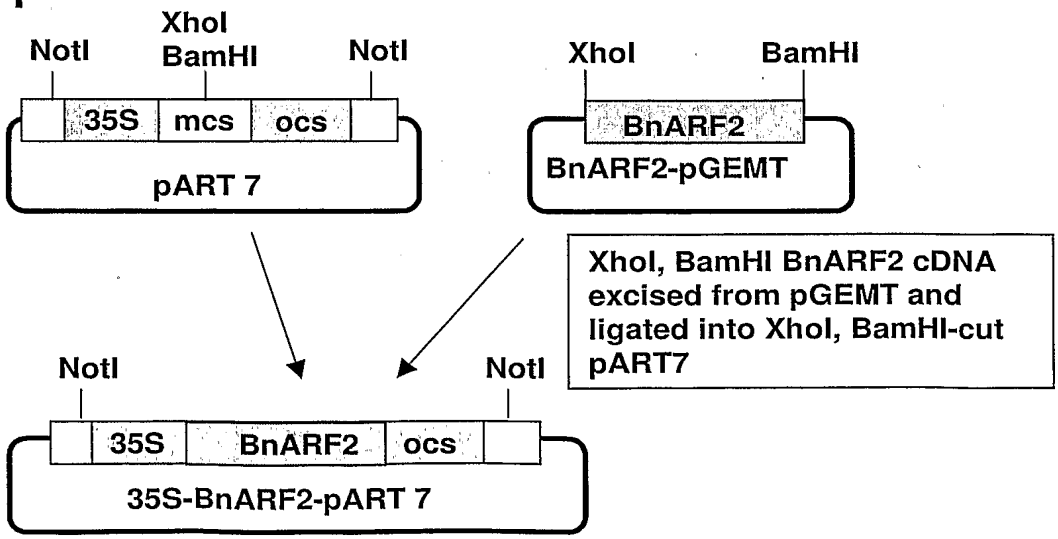


Figure 17
Cloning strategy, Examples 8, 9

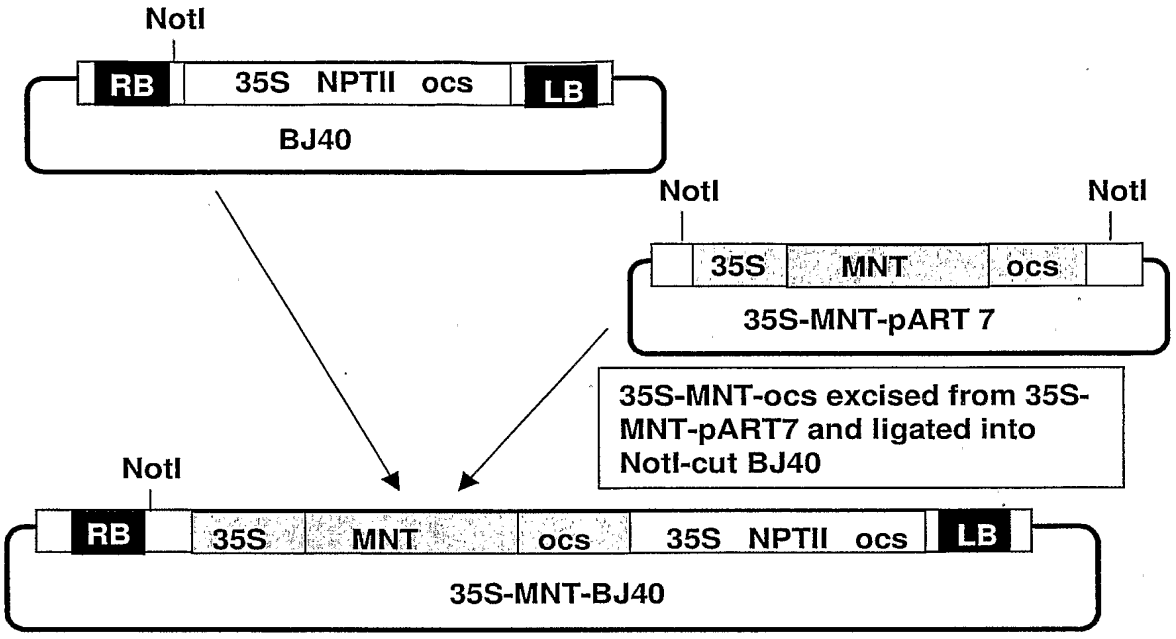
Example 8a



Example 9a



Example 8b



Example 9b

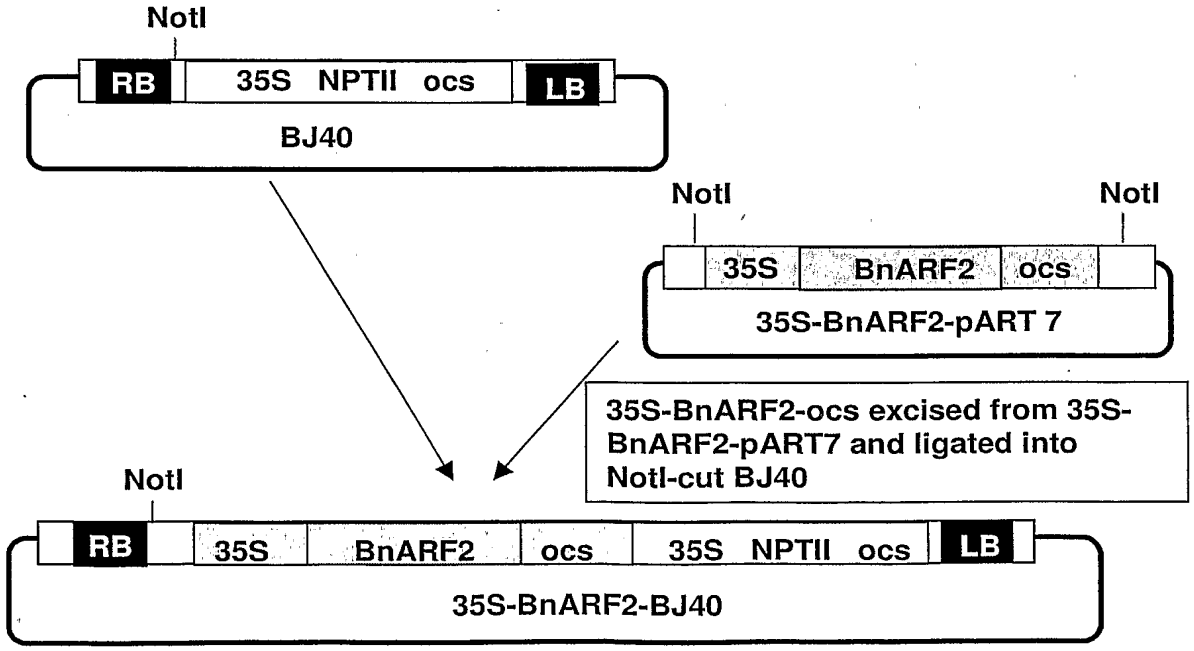
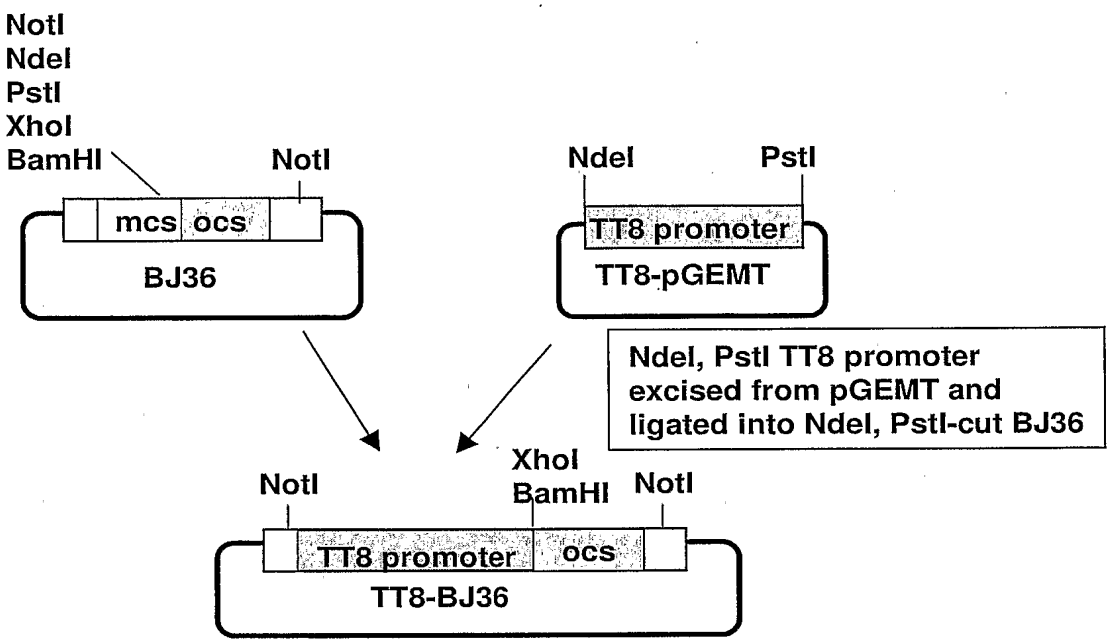


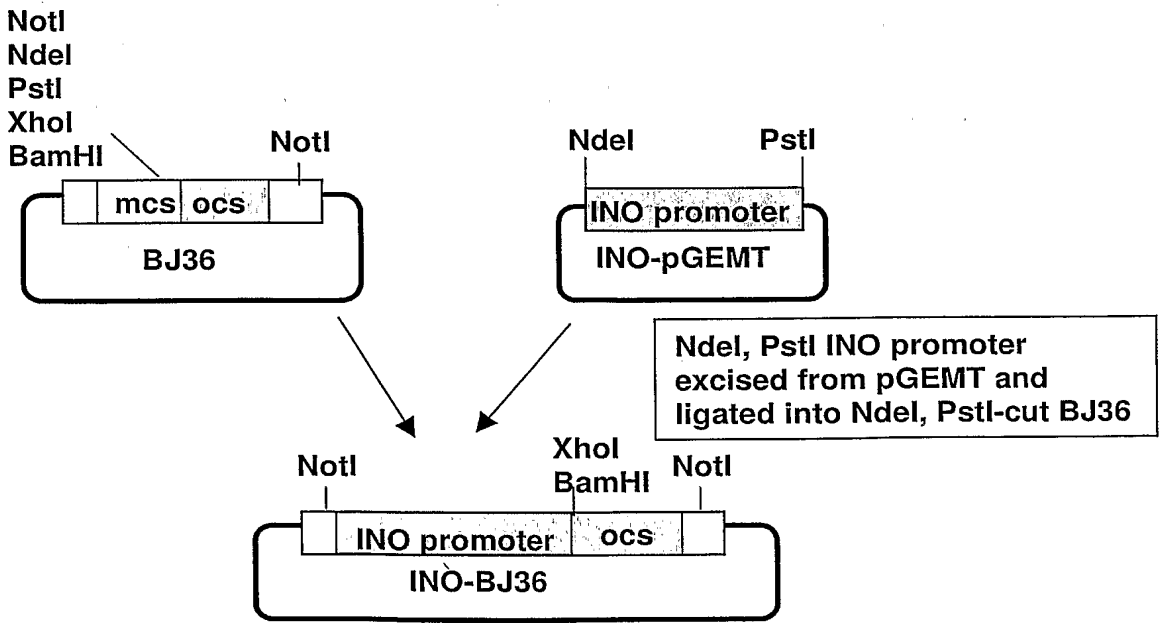
Figure 18

Cloning strategy, Example 10

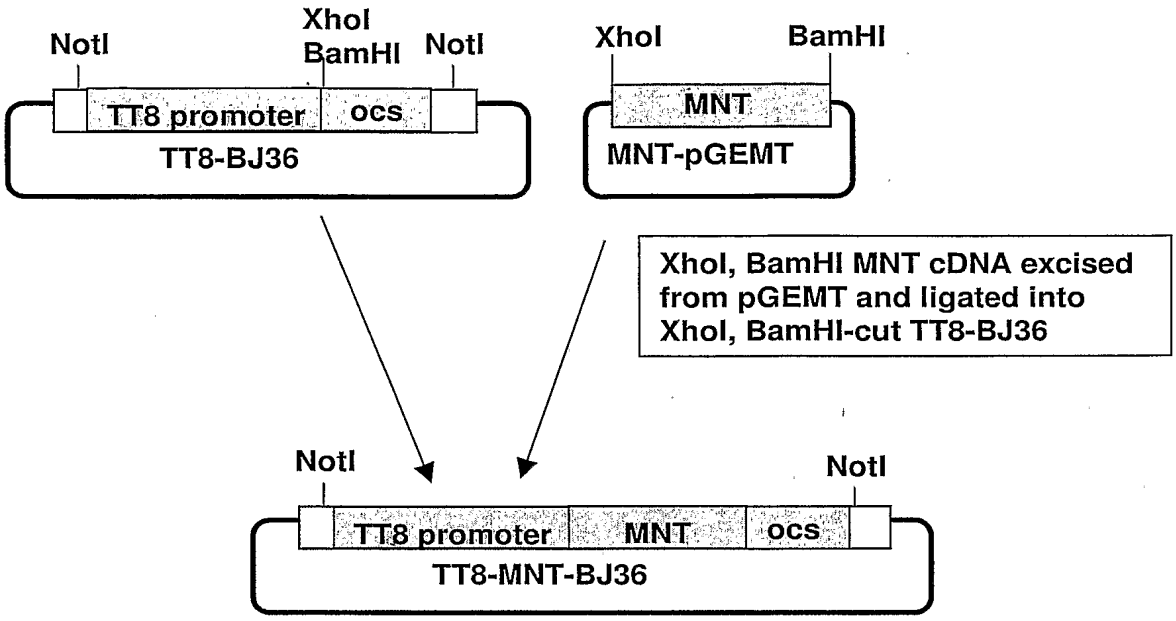
Example 10a(i)



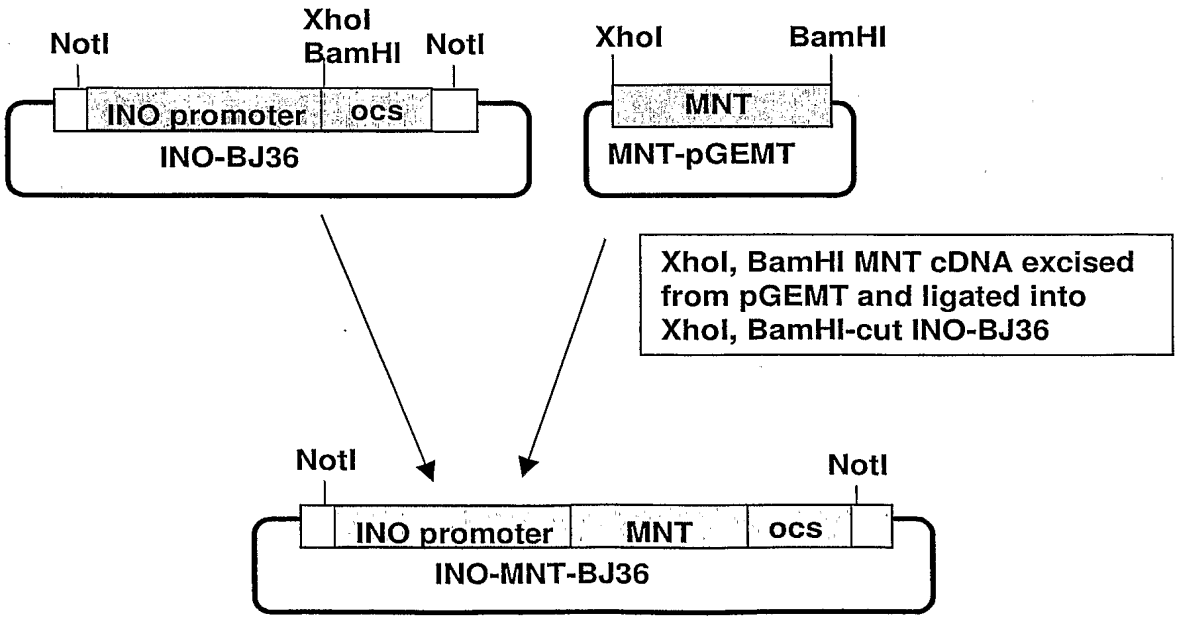
Example 10a(ii)



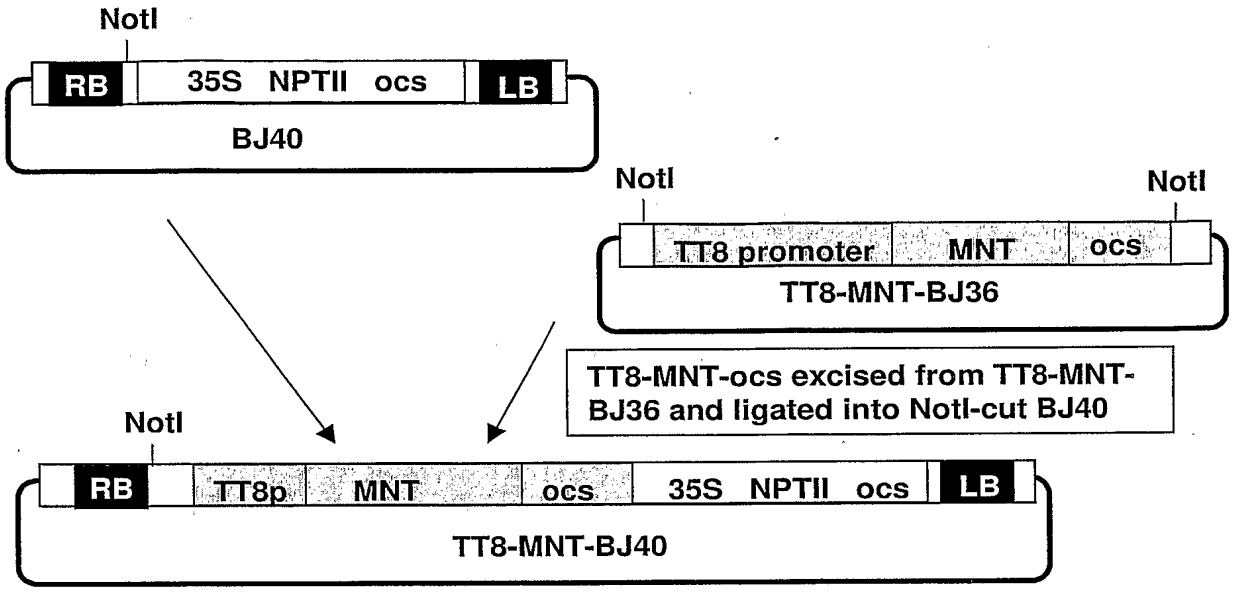
Example 10b(i)



Example 10b(ii)



Example 10c(i)



Example 10c(ii)

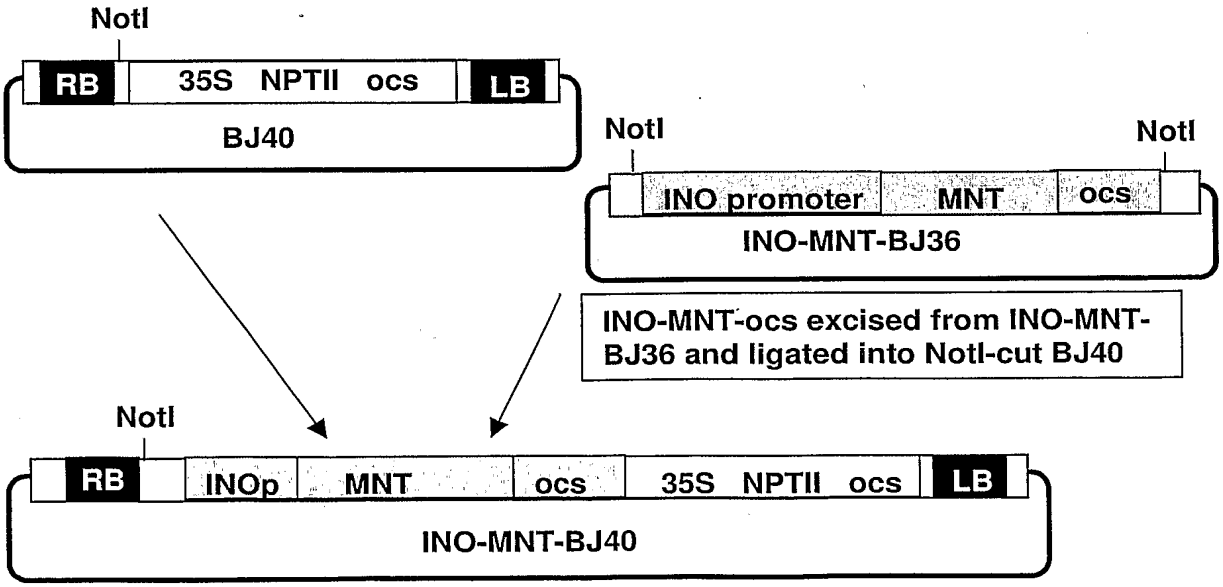
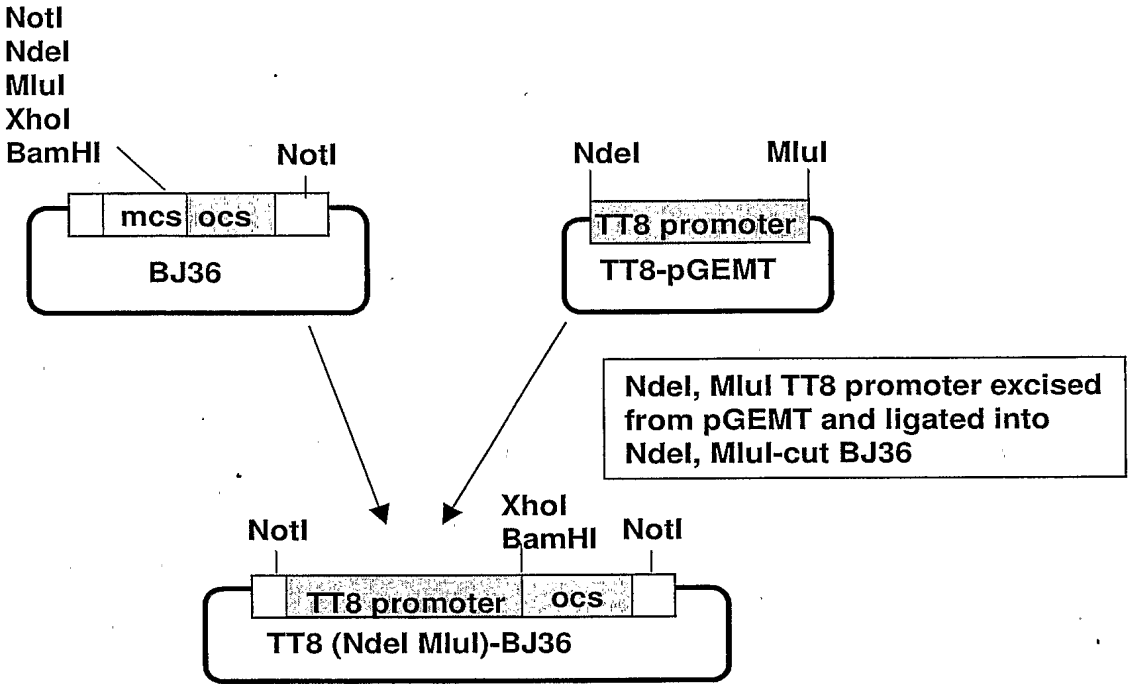
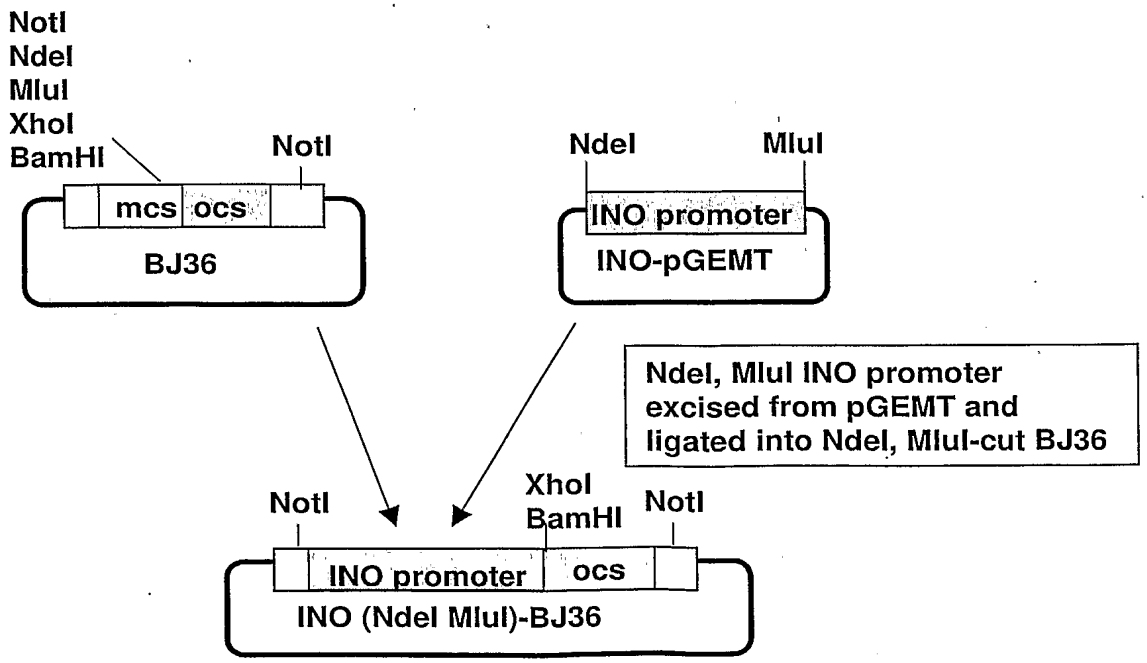


Figure 19
Cloning strategy, Example 11

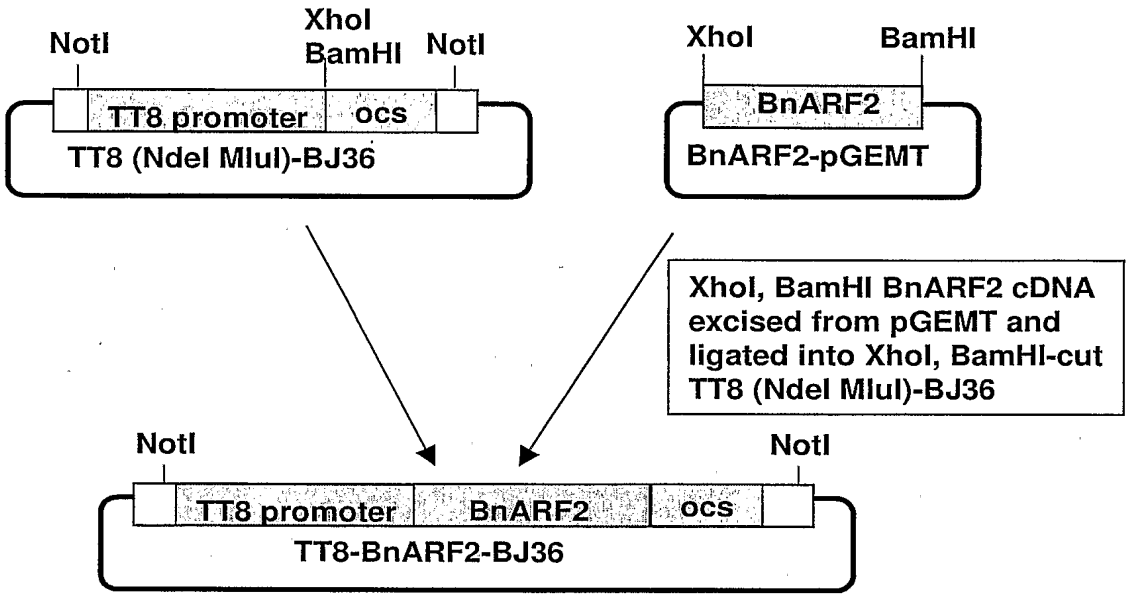
Example 11a(i)



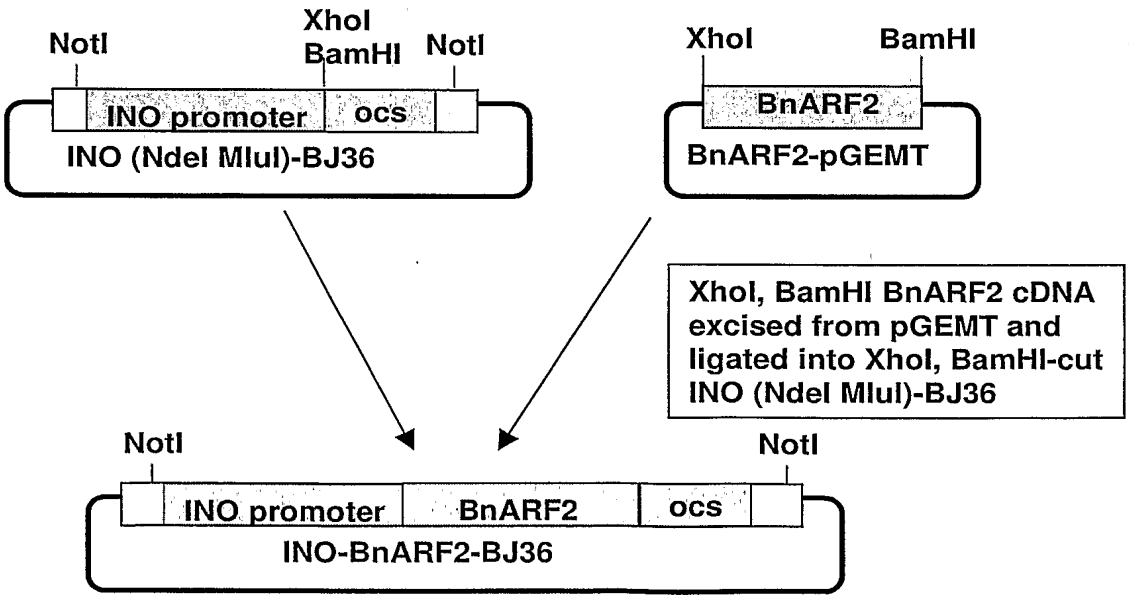
Example 11a(ii)



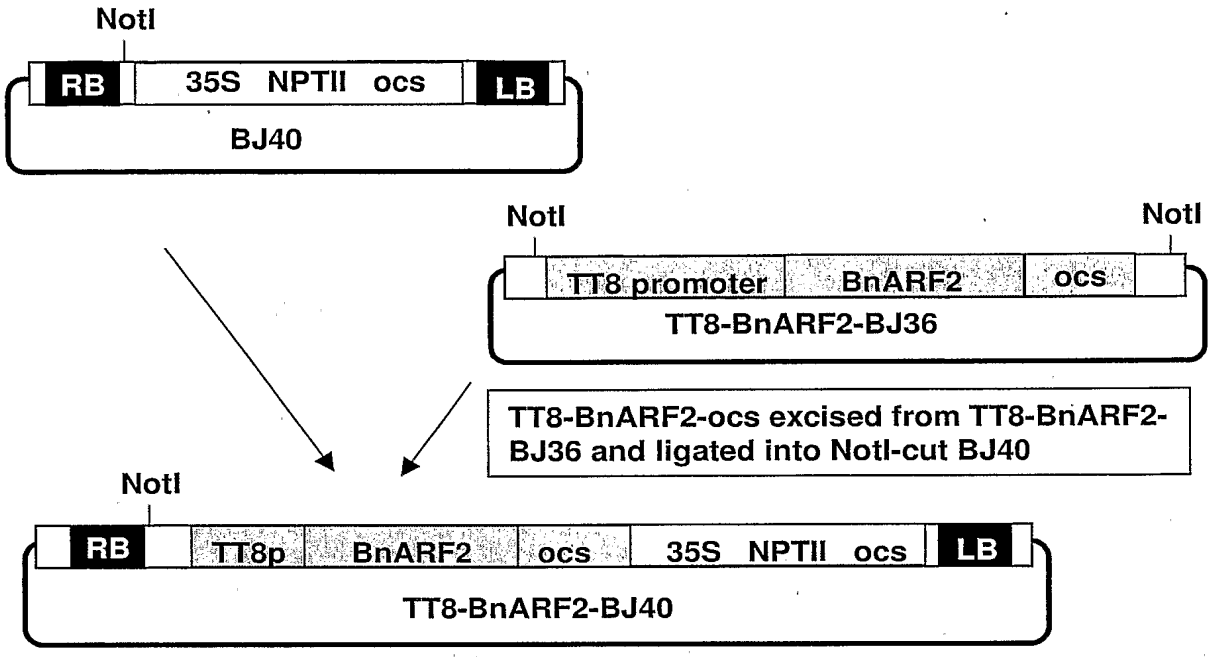
Example 11b(i)



Example 11b(ii)



Example 11c(i)



Example 11c(ii)

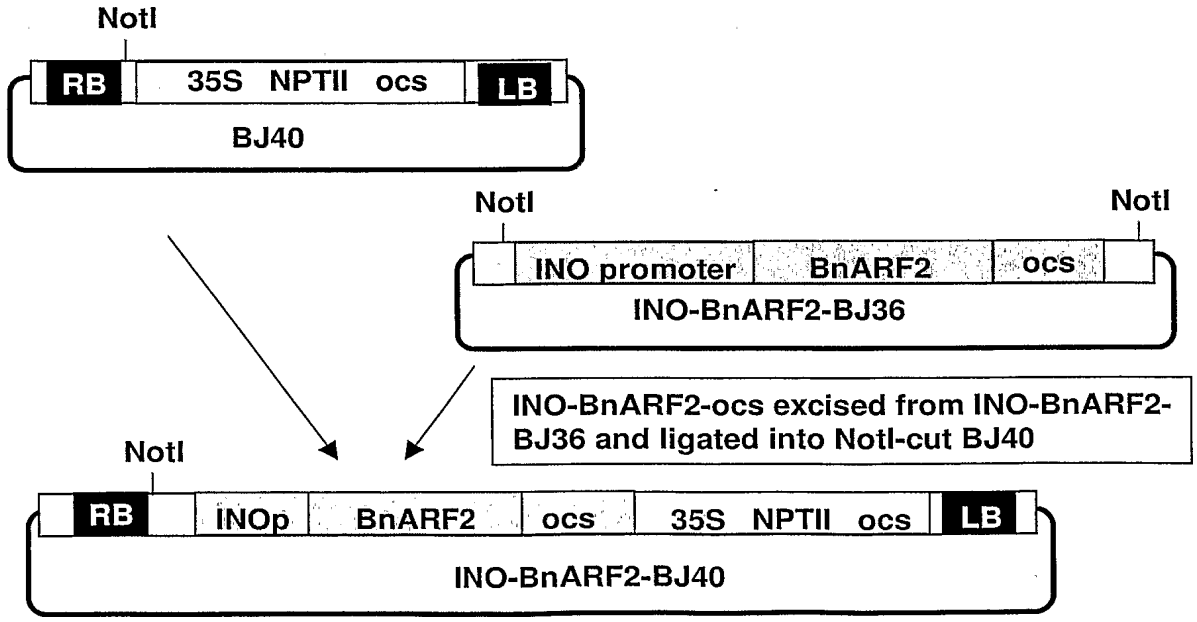
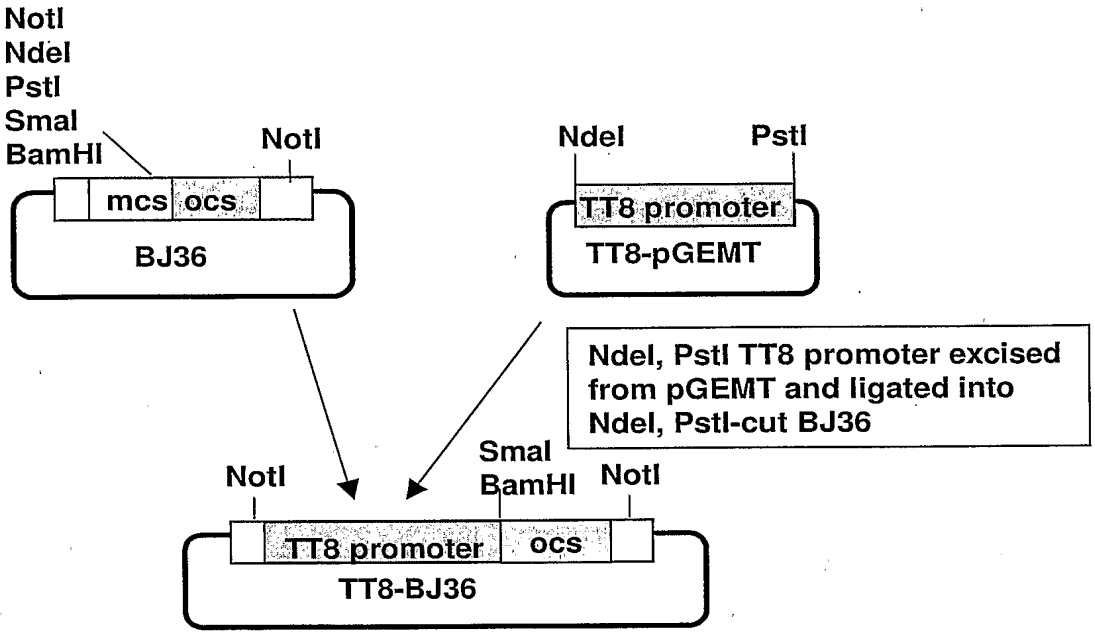


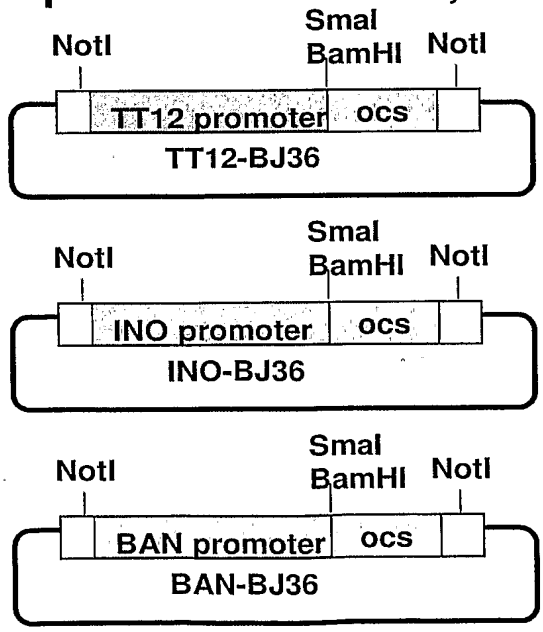
Figure 20

Cloning strategy, Examples 12, 13

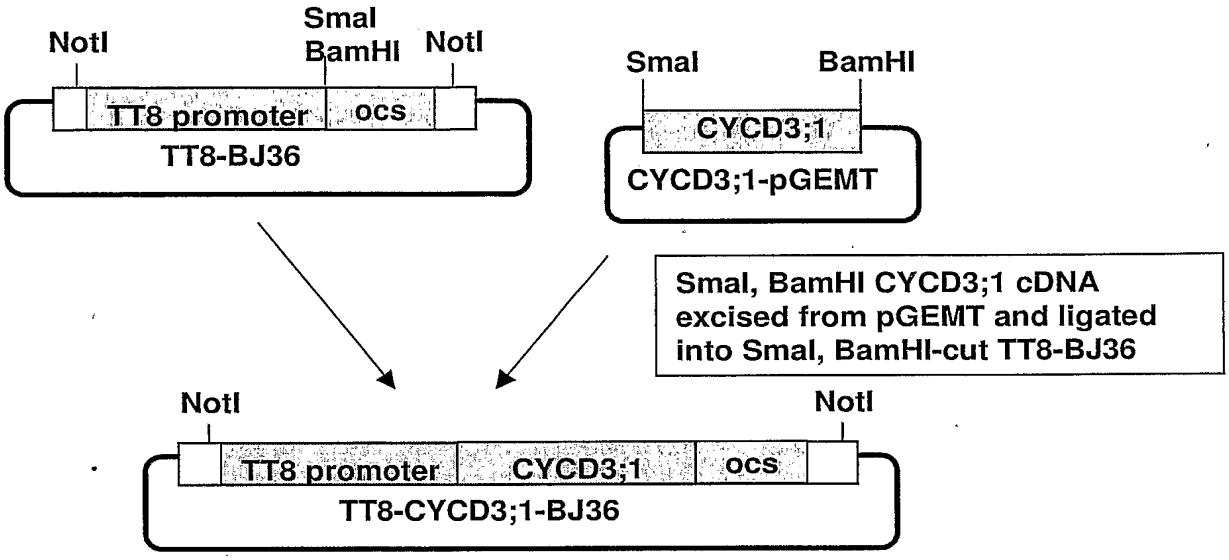
Examples 12a, 13a



Repeat process with TT12, INO, BAN promoters



Examples 12b, 13b



Repeat process with IPT1, ANT, CYCB1;1 cDNAs and TT12, INO, BAN promoters

TT8-IPT1-BJ40

TT8-ANT-BJ40

TT8-CYCB1;1-BJ40

TT12-CYCD3;1-BJ40

TT12-IPT1-BJ40

TT12-ANT-BJ40

TT12-CYCB1;1-BJ40

INO-CYCD3;1-BJ40

INO-IPT1-BJ40

INO-ANT-BJ40

INO-CYCB1;1-BJ40

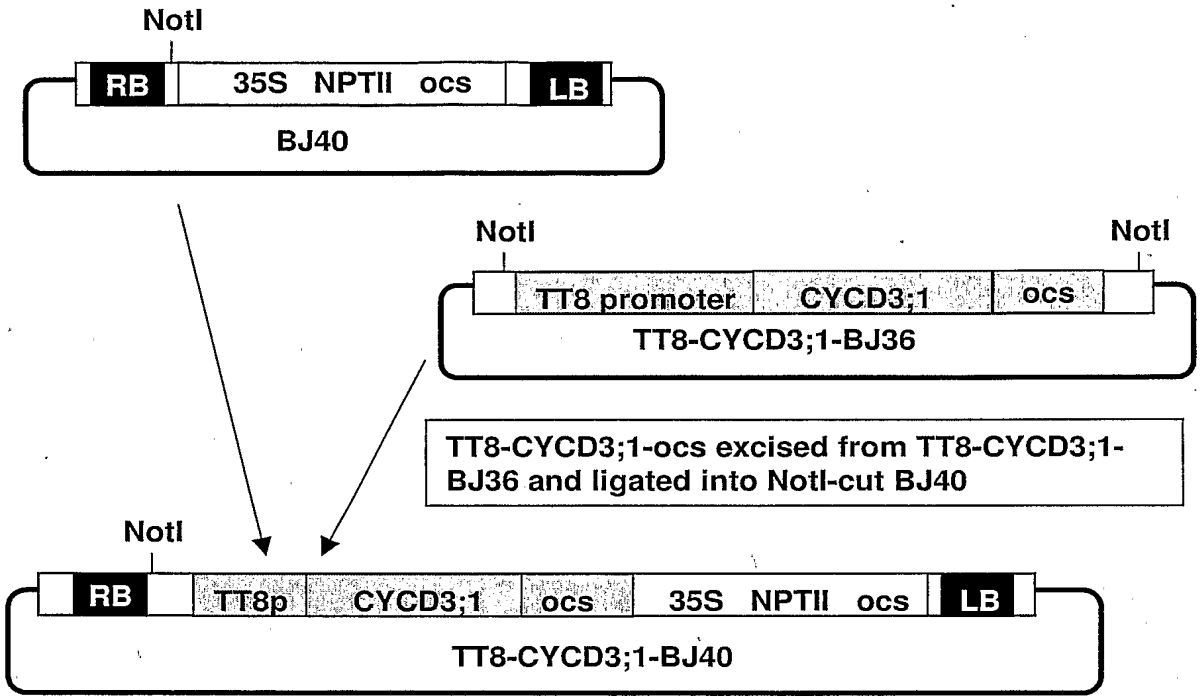
BAN-CYCD3;1-BJ40

BAN-IPT1-BJ40

BAN-ANT-BJ40

BAN-CYCB1;1-BJ40

Example 12c, 13c



Repeat process with all BJ36 constructs shown in Example 12b

TT8-IPT1-BJ40

TT8-ANT-BJ40

TT8-CYCB1;1-BJ40

TT12-CYCD3;1-BJ40

TT12-IPT1-BJ40

TT12-ANT-BJ40

TT12-CYCB1;1-BJ40

INO-CYCD3;1-BJ40

INO-IPT1-BJ40

INO-ANT-BJ40

INO-CYCB1;1-BJ40

BAN-CYCD3;1-BJ40

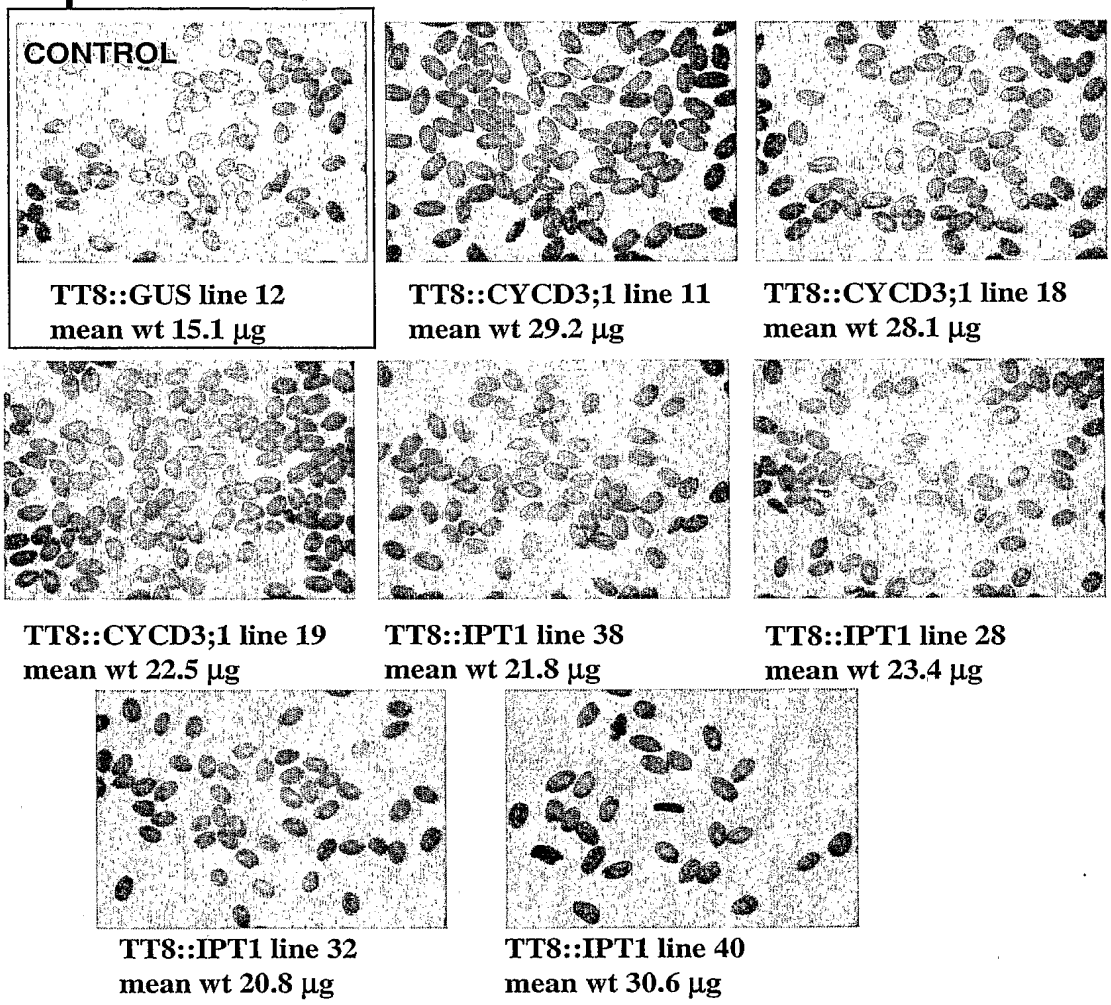
BAN-IPT1-BJ40

BAN-ANT-BJ40

BAN-CYCB1;1-BJ40

Figure 21

Expression cassettes to increase seed size



Distribution of seed weights in TT8::GUS (control), TT8::CYCD3;1, and TT8::IPT1families

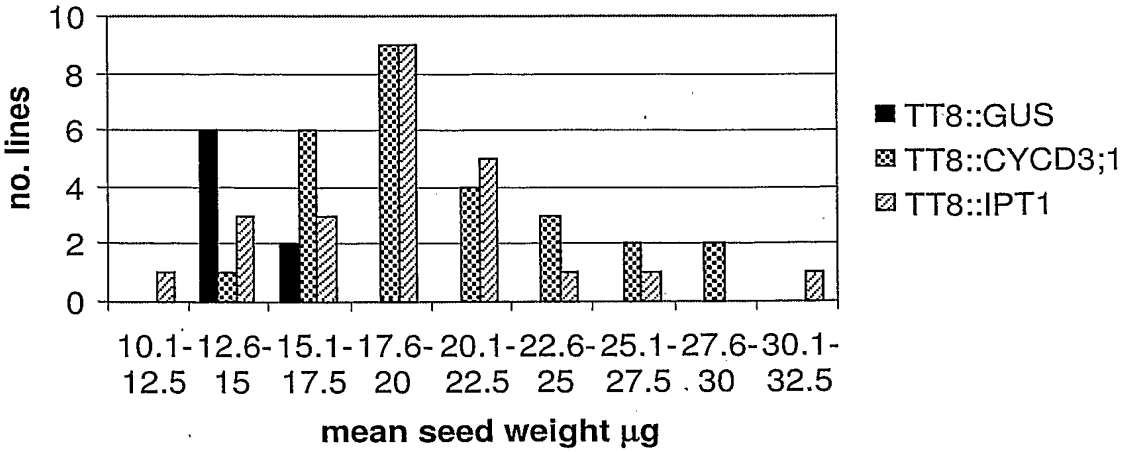
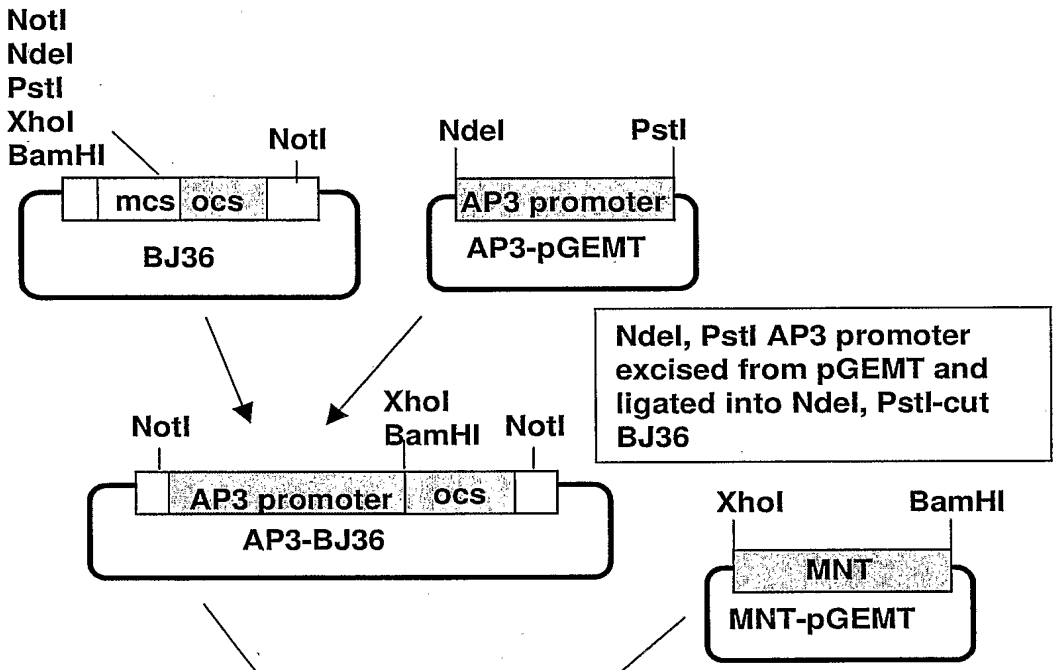
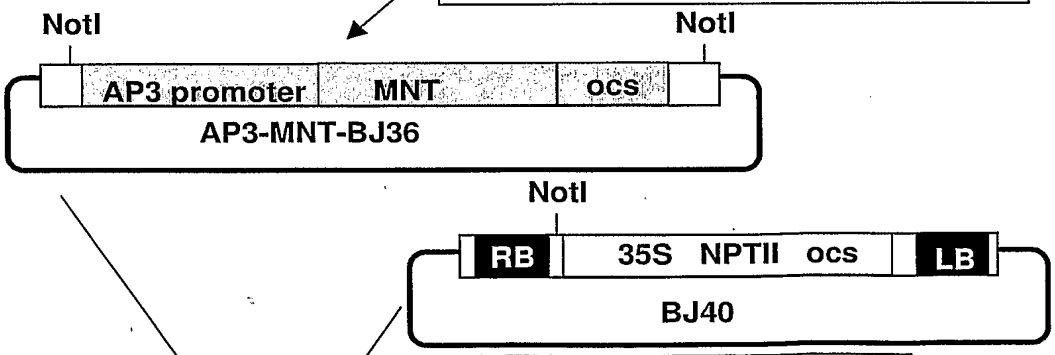
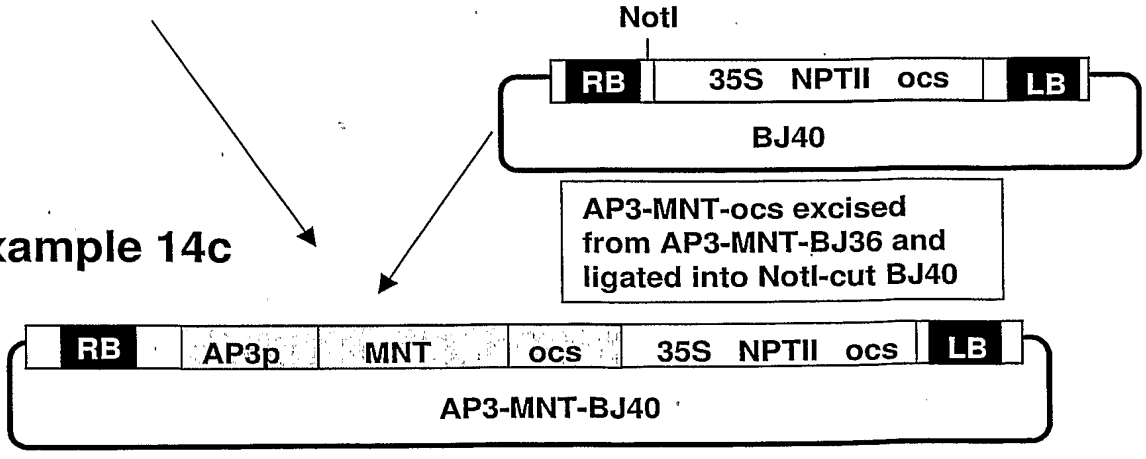


Figure 22
Cloning strategy, Example 14

Example 14a



Example 14b



Example 14c